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Corynebacterium diphtheriae and Its Relatives[1]

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[1]Although the documentation supporting the point of view presented here amounts to a review of relevant literature, no effort has been made to include in this paper an exhaustive listing of papers related to *C. diphtheriae*.

[2]Drawings by James E. Ziegler.

To André Lwoff, artist and young man, in his fiftieth year in science and to Hidebumi-san, Mak-san, and A. M. P., Jr.

INTRODUCTION

From November to March in the northern hemisphere (25, 165, 319) and from April to August in the southern hemisphere, wherever there are large numbers of human beings, infections with *Corynebacterium diphtheriae* are likely to occur. Several cases have been reported in the southeastern U.S. as recently as 1969 (31). [In the summer of 1970 there were at least two outbreaks of diphtheria in the United States: one in San Antonio, Tex., and one in Washington, D.C.] Although diphtheria must have plagued man since ancient times, it was not until 1826 when Pierre Brettoneau described the clinical entity, diphtheria, involving the appearance of a pseudomembrane in the throat, that meaningful recording of this disease began. After the etiological agent(s), *C. diphtheriae*, had been discovered by Klebs in 1883 (161) and related to the disease by Loeffler in 1884 (185), a rational means was available for distinguishing diphtheria from other maladies of the throat. In 1888, Roux and Yersin (265) made the exciting discovery that toxic filtrates could be easily obtained free of diphtheria bacilli and that these filtrates were lethal for animals. Since the symptoms produced by toxin in animals (neurologic changes, cardiac failure, etc.) accounted for the more dramatic signs seen in human diphtheritic infections, the action of toxin was held to account for the pathogenesis of diphtheria, and diphtheria bacilli which did not produce toxin were all but ignored and their relationship with *C. diphtheriae* was little considered. In fact, for many years it was erroneously assumed that toxin was required for pseudomembrane formation (62).

When Freeman discovered in 1951 that certain bacteriophages could endow nontoxinogenic *C. diphtheriae* with the capacity to produce toxin (81), interest in the biology of diphtheria and that of its etiologic agents was rekindled. Soon one of the temperate phages carrying the gene *tox*, β^{tox^+}, was characterized and pairs of toxinogenic and nontoxinogenic strains of *C. diphtheriae*, such as $C7_s(-)^{tox^-}$, $C7_s(\beta)^{tox^+}$, $C4_s(-)^{tox^-}$ and $C4_s(\beta)^{tox^+}$, were isolated, cloned, and studied (27). For the first time in the 68 years since their discovery, diphtheria bacilli differing by only one prophage (gene) could be compared. It was at once obvious that invasiveness (virulence) and toxinogenicity were separable properties (23, 116, 215). For example, rabbits infected with $C7_s(-)^{tox^-}$ developed pseudomembranous lesions but later recovered from their infections, whereas rabbits infected with $C7_s(\beta)^{tox^+}$ developed necrotic lesions and died (23). This separation of invasiveness from toxinogenicity was consistent with the reported cases of diphtheritic infections in man caused by nontoxinogenic diphtheria bacilli as well as those reports of diphtheria in individuals having circulating antitoxin (23, 31, 72, 134). The relationship between the easily distinguishable properties invasiveness (virulence) and toxinogenicity to the etiology of toxaemic and nontoxaemic diphtheritic infections is clearly illustrated in Fig. 1.

Although it was the disease diphtheria that led to the discovery of *C. diphtheriae*, this article will be concerned principally with the bacterium *C. diphtheriae* as the type species of the genus *Corynebacterium*.

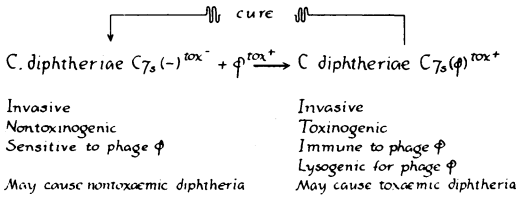


FIG. 1. *Changes brought to $C7_s(-)^{tox^-}$ after lysogenization by a bacteriophage carrying the tox gene. Presumably the indicator strain $C7_s$ is nonlysogenic, hence the designation (−), and is nontoxinogenic, tox^-. When nontoxinogenic, nonlysogenic $C7_s$ is lysed by a phage carrying the tox^+ marker, such as ϕ^{tox^+}, toxin is produced during the course of phage multiplication and lysis of the cell. When lysogenized by ϕ^{tox^+}, the genome of $C7_s$ $(\phi)^{tox^+}$ includes phage genes which endow it with immunity to homologous phage (lysogenic immunity = synthesis of specific repressor) and the ability to synthesize diphtherial toxin. The subscript s refers to the smooth (surface) antigen of the strain. Once the K antigens of Lautrop (see text) are systematized, s would be replaced with a more specific designation, e.g. $C7_{K15}(-)^{tox^-}$. For other cases of changes in bacteria brought about by the presence of prophage, see Fig. 15 and 16.*

CORYNEBACTERIUM, MYCOBACTERIUM, AND NOCARDIA: THE CMN GROUP

The genus *Corynebacterium* as conceived by Lehman and Neuman (1896) is synonymous with *Corynethrix* of Czaplenski (1900), *Corynemonas* of Orla-Jensen (1909), and *Corynobacterium* of Enderlein (1917). The type species was designated *Corynebacterium diphtheriae* (Flügge, 1886) Lehmann and Neumann, 1896, by Winslow et al. (180). The Winslow committee described the genus as "slender, often slightly curved, rods with a tendency to club and pointed forms, branching cells reported in old cultures. Barred uneven staining. Not acid fast. Gram-positive. Aerobic. No endospores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide" (315). It is worth noting that each of these authors suggested a close relationship between *Corynebacterium* and the actinomycetes. Despite this suggestion, in the intervening years *Corynebacterium* was removed from the actinomycetes and a number of unrelated or distantly related organisms was placed in the genus simply because, on morphological grounds, they could be made to fit the general description. However, the intuitive feeling of the original authors and of the Winslow committee that *Corynebacterium*, *Mycobacterium*, and *Nocardia* were closely related has been amply borne out in recent years through the accumulation of information regarding the molecular constituents of the envelopes of members of these three genera.

The mureins [(307) peptidoglycans, muropeptides] of their cell walls contain combinations of related molecular species not found in the walls of a number of pleomorphic gram-positive bacilli such as those group G streptococci called *C. pyogenes* (26, 59), the "plant pathogenic corynebacteria" (234, 235) or those propionibacteria (66) called *C. acnes*, *C. parvum*, *C. avidum*, etc. (218, 309). It is evident, therefore, that morphology is a poor criterion on which to relate bacteria. Many bacteria become pleomorphic under conditions that make for unbalanced cell wall synthesis. One of the first examples of this kind of phenotypic modification was that of *C. diphtheriae* growing on a nutritionally inadequate medium, the inspissated serum slopes of Loeffler. Such bacilli developed a number of thin spots in their walls with consequent swelling and bulging. Some of the shapes they assume were described as clubs, giving rise to the name *Corynebacterium* (clubbacterium). Certainly, this was a more fortunate choice than say, rendering into Latin the "likeness to Chinese characters" which some writers ascribe to diphtheria bacilli. In Fig. 2E is shown a pair of cells of *C. diphtheriae* which are from a culture in the logarithmic phase of growth. These cells have just separated after completion of septum formation. They show a characteristic tapering towards the end distal to the septum. Figure 3 illustrates cells of *C. ulcerans*, *C. ovis*, *C. diphtheriae*, *Propionibacterium acnes*, and *Streptococcus viridans*. These are shown because they illustrate the point that some of the bacilli look like cocci and some of the cocci look like "coryneform" or "diphtheroid" bacilli. Despite the apparent meaninglessness of morphology as a parameter of taxonomic value in this case, taxonomists continue to wrestle with the "coryneform" tangle as though the phenotypic variations responsible for its seeming reality actually have a common molecular origin (142, 318). Since the wall structure is widely different in the case of *Corynebacterium*, *Propionibacterium*, and *Streptococcus*, it follows that the basis for pleomorphism in each case is different. It is the *basis itself* rather than the pleomorphism that is of taxonomic value. In this regard, the terms "coryneform" and "diphtheroid" would seem today of little use in the characterization of bacteria (*see* Fig. 4). It is recommended that they be dropped.

Cummins and Harris (57–60) have shown that the cell walls of what this writer takes to be legitimate members of the genera *Corynebacterium*, *Mycobacterium*, and *Nocardia* have in common a muramyl peptide containing *meso*-α,ϵ-diaminopimelic acid (DAP), glutamic acid, and alanine in association with arabinogalactan and that the walls of cells from these genera show serologic relatedness (58). On the other hand, preparations of envelopes of organisms of such wrongly named species as *C. pyogenes* and *C. betae*, which have rhamnosyl units in their wall polysaccharide and lysine substituted for DAP in their muramyl peptide, do not react with sera prepared against walls of the *CMN* group. In addition to having basically similar mureins (307), the *CMN* organisms incorporate into their walls corynemycolic (2-tetradecyl-3-hydroxystearic acid, $C_{32}H_{64}O_3$) and corynemycolenic (2-tetradecyl-3-hydroxy-9-octanedecanoic acid, $C_{32}H_{62}O_3$) acids of *C. diphtheriae* or tetrahydronocardic acid ($C_{50}H_{96}O_3$) of *N. asteroides* (199) or the mycolic acids of *M. tuberculosis* (12) which are as much as 88 carbons ($C_{88}H_{176}O_3$) in length (10). These mycolic acids have been found in ester linkage with arabinose (of the arabinogalactan) in mycobacteria (16, 40, 139, 145–147, 202, 236) and in *Nocardia* (173), but not yet in *Corynebacterium* (10, 11, 78, 177, 308). In mycobacteria such mycosides were first found as a

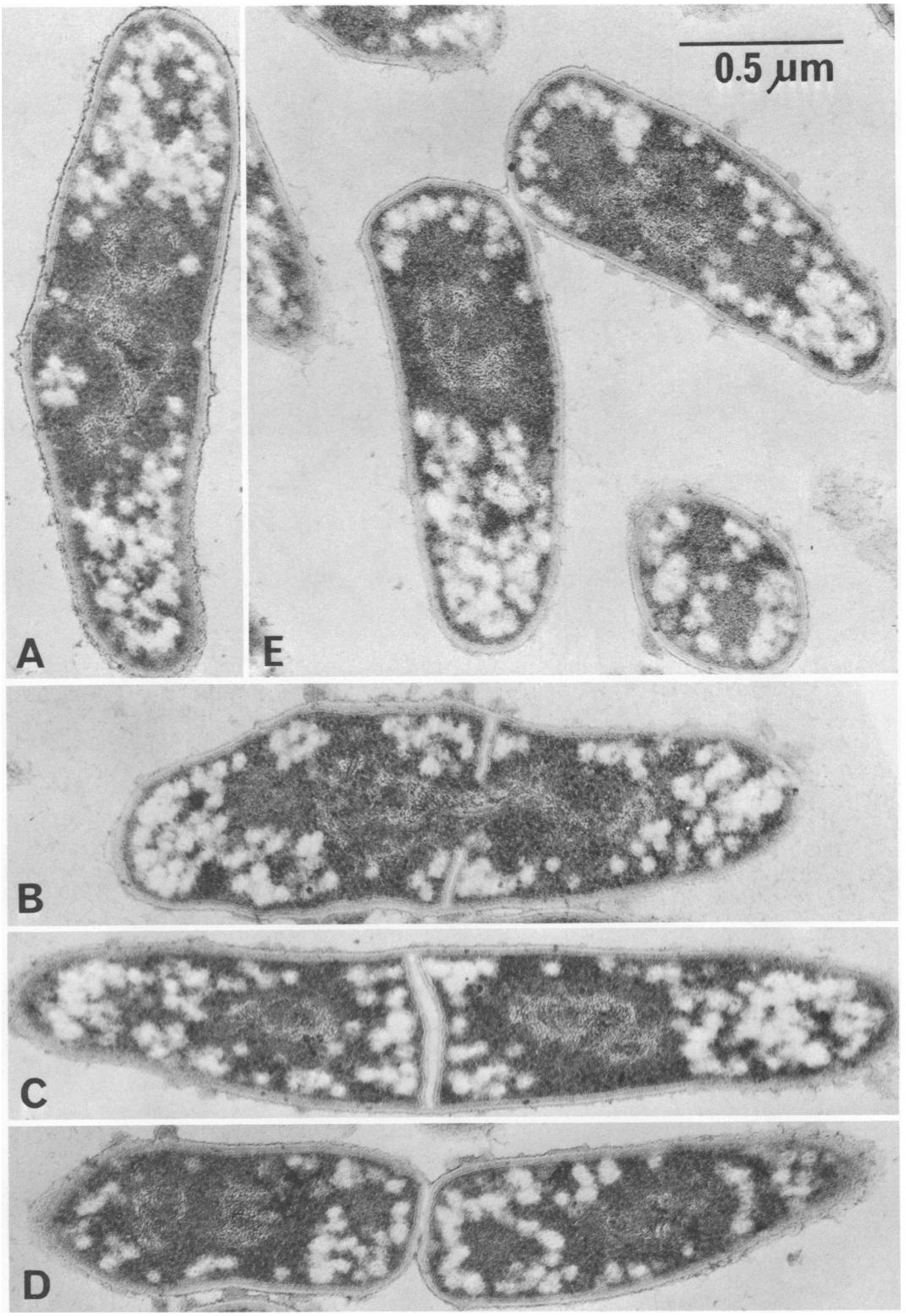


FIG. 2. *C. diphtheriae strain C7$_s$(−)$^{tox^-}$ under conditions allowing for growth at maximal rate. (A) Initiation of septum formation by ingrowth of membrane. (B) Well-developed septal initials showing "layers" of the components of the cell envelope. (C) Two cells still connected showing that the septum consists of two full complements of membrane and envelope components. (D) Beginning of separation of cell doublets. (E) The "snapping" involved in the pulling apart of two corynebacterial cells, showing the characteristic taper from septal to distal end. Electron-opaque areas, peculiar to actively growing cells, seem not to be glycogen but may represent lipid associated with loci of intense biosynthetic activity. × 51,000. Bar = 0.5 μm. From data of Sheila Heitner assembled by Kwang Shin Kim.*

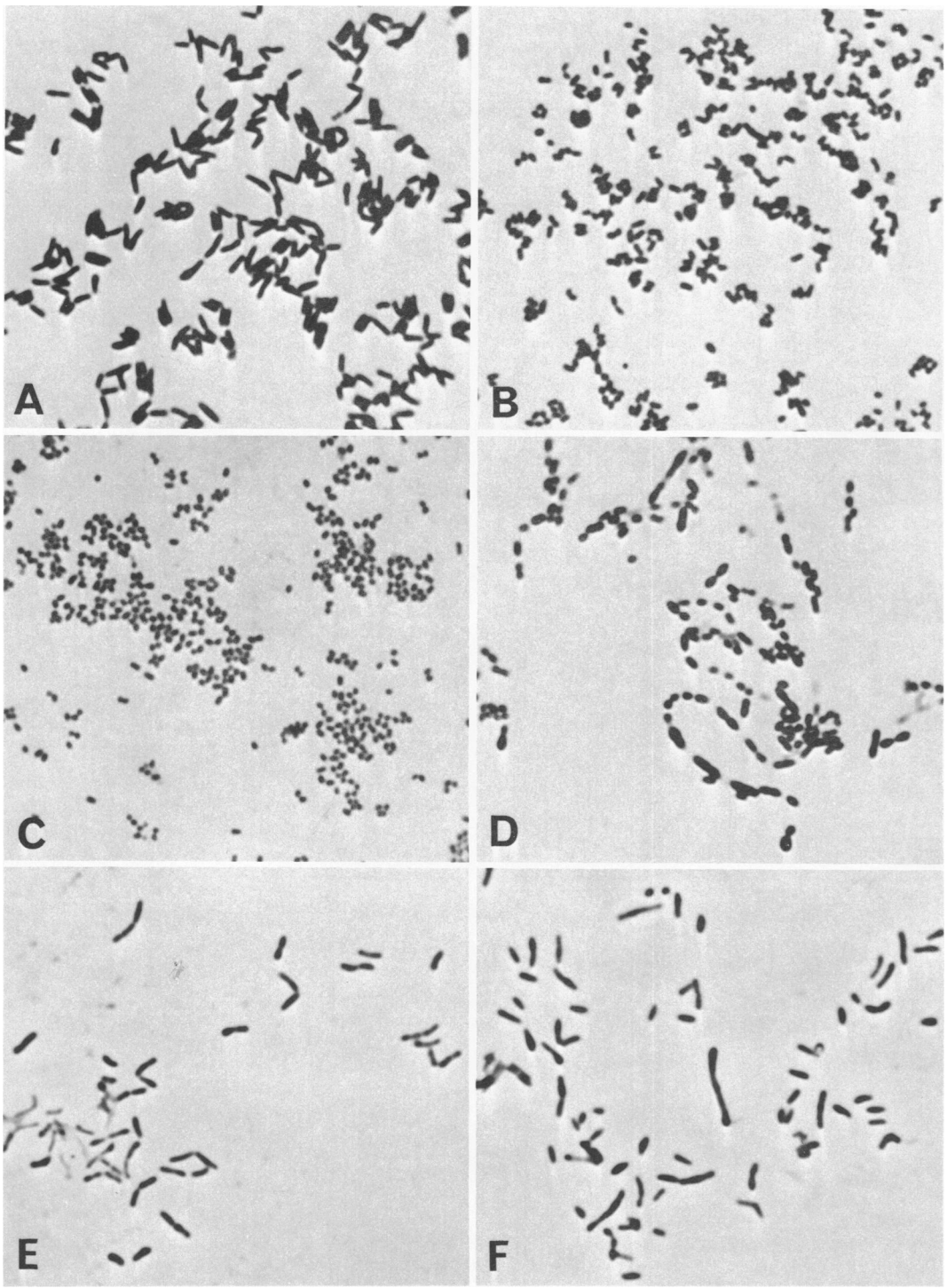


FIG. 3. *Smears stained with the Gram stain: A, Cells of C. diphtheriae, strain C7$_s$(−)$^{tox^-}$; B, C. diphtheriae var. ulcerans; C, C. pseudotuberculosis (ovis); D, Streptococcus viridans. Phase-contrast micrographs: E, Propionibacterium sp. strain 2629 LT; F, Mycobacterium strain NQ. Pictures by K. S. Kim.* × 4,000

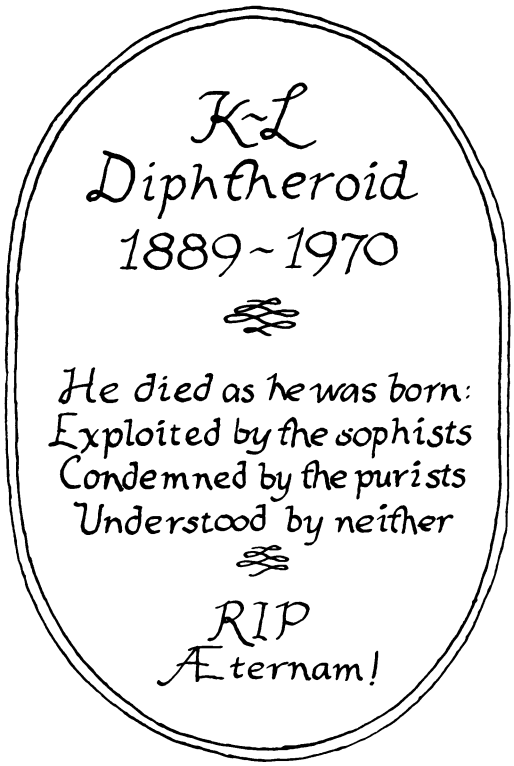


FIG. 4. *Plea for revision of our lexicon for corynebacteriology.*

part of the well-known peptidoglycolipid Wax D (10), a fragment of the mycobacterial envelope consisting of two *meso*-DAPs, two L-alanines, one D-alanine and two D-glutamic acids in amide linkage with galactosamine glycosidically linked to arabinogalactan (plus a mannose-, glucosamine-, and galactosamine-heteropolymer), linked to a ground substance moiety of glucosamine, muramic acid, alanine, and *meso*-DAP from one part of the chain and in ester linkage with mycolic acids from another. [Azuma, Ajisaka, and Yamamura (16*a*) have now embarked on an exploration of the polysaccharides of mycobacteria and their relationship to those of *C. diphtheriae* and *Nocardia asteroides*. In addition to arabinogalactans, the polysaccharides so far studied include arabinomannan, mannan, and glucan.] The use of a collection of enzymes for selectively breaking specific bonds between the subunits of bacterial cell walls (84) in conjunction with analyses of the isolated subunits by mass spectrometry has made possible a considerable accumulation of data on the peptides present in walls of members of the *CMN* group. *Mycobacterium* and *Corynebacterium* contain the diamidated tetrapeptide L-Ala$^{\gamma}$-Gln-*meso*-DAP-(NH_2)-D-Ala. In mycobacteria and corynebacteria, at least, Glu- and D-Ala are linked to the same asymmetric carbon of DAP and the amide groups of the diamidated "tetrapeptides are located on Glu- and on the asymmetric carbon of DAP not linked to Glu" (311). It seems safe to assume that variations on this pattern are to be found throughout the *CMN* group (Fig. 5). Azuma and others have just now shown that *M. smegmatis*, *M. kansasii*, *M. tuberculosis* BCG, and *M. phlei* have N-glycolylmuramic acid in their mureins in place of the more usual N-acetyl derivative (1, 17, 147). Guinand and Michel had previously reported the presence of *N*-glycolylmuramic acid in *N. kirovani* (98). Kanetsuna and San Blas (147) suggested that in the cell envelopes of strain BCG and *M. smegmatis* ATCC 14468 part of the linkages between arabinogalactan and muropeptide is via N-glycolated muramic acid and part via phosphodiester bridges (182). Whether true corynebacteria with N-glycolated peptidoglycan will be found remains to be seen.

It has recently been shown that cultures of *M. tuberculosis*, $H_{37}Rv$, treated with cycloserine, accumulate an arabinogalactan-galactosamine-DAP-mycolate (AGDM) which, under ordinary circumstances in modified form, would probably be incorporated into the cell envelopes of these organisms (61). Presumably D-cycloserine, here as with other microorganisms, interferes with both the racemase (266) responsible for the conversion of L-alanine to D-alanine and the synthetase (217) which catalyzes the bonding that yields D-alanyl-D-alanine. Since alanyl peptides form a key part of the linkage of murein to the glycolipids of the cell envelope, blocking of their synthesis could very well result in the liberation of AGDM into the medium.

Members of the *CMN* group behave as adjuvants when administered to animals with immunizing agents, and lipid-containing fractions such as Wax D also are themselves good adjuvants (10). Dimycolates of α,α'-trehalose, the cord factors, related to surface properties of these bacteria and to their virulence, have been described for *Corynebacterium* (248) and *Mycobacterium* (221) but not yet for *Nocardia*. A possible relation to the cell envelope of the various molecular species discussed is diagrammed in Fig. 5.

Although detailed metabolic studies have not been carried out on many members of the *CMN* group, it is evident from studies that have been made that *CMN* organisms have much in common

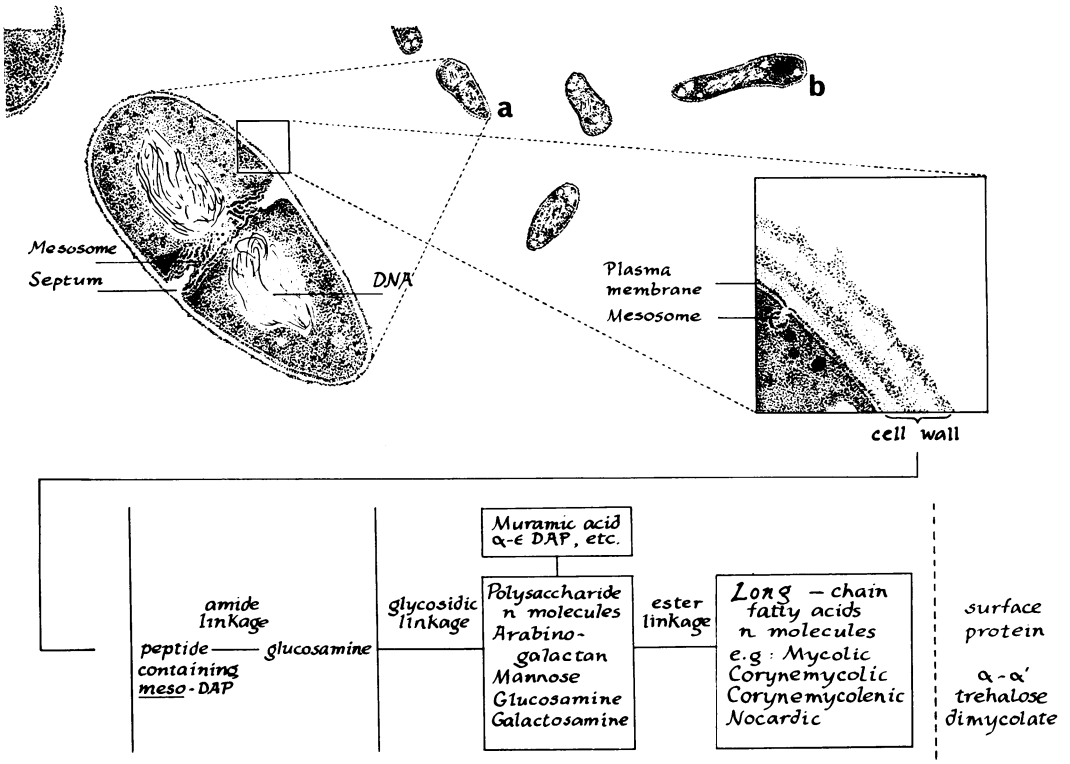


FIG. 5. *(a) Diagramatic sketch of an actively growing bacterial cell representing a composite of the CMN group. (b) A "Resting" cell with metachromatic granule. A portion of the envelope of an actively growing cell (a) has been expanded to show the relation of the complex envelope to the cytoplasmic membrane. A portion of the wall is shown to consist of murein, arabinogalactan mannan linked to species of long-chain, α-branched, β-hydroxylated fatty acids, the mycolic acids, and to dimycolates of trehalose and to a surface protein antigen. The mureins and arabinogalactans are distinctive from genus to genus as are the mycolic acids: e.g., mycolic (Mycobacterium), corynemycolic and corynemycolenic (Corynebacterium), and nocardomycolic (Nocardia). In general terms the murein-arabinogalactan is a heat-stable O antigen; the heat-labile surface protein antigen is the K antigen. For actual dimensions relating to these anatomical components, see rigid (murein) layer in Fig. 6–8; surface layers in Fig. 6, 7, 9, and 10. For further details, see the text and the following references: 2, 15, 16a, 68, 87, 156, 159, 160, 171, and 289.*

with regard, for example, to storage or utilization, or both, of polyphosphate (2, 68), the accumulation of Sudan Black B-positive fat (42), the synthesis of a special class of menaquinones (29, 277) which are used in electron transport (9, 32, 38, 276, 296), similar overall patterns of respiration (9, 38, 167), as well as the synthesis of a common class of long-chain, α-branched, β-unsaturated fatty acids, the mycolic acids (10). The differences which separate corynebacteria, mycobacteria, and nocardias include nutritional requirements, mean generation times, and acid-fastness. In addition, the sulfolipid isolated by Middlebrook, Coleman, and Schaefer (200) from neutral red-binding, virulent *M. tuberculosis* strain $H_{37}Rv$ and shown by Middlebrook (200) and others (130) to be associated with many virulent strains of *M. tuberculosis*, has not yet been reported in either *Nocardia* or *Corynebacterium*. Mayer B. Goren has shown that the neutral red-binding strain, $H_{37}Rv$, produces not one but several families of structurally related sulfolipids of which Sulfolipid I is a complex glycolipid ester with a molecular weight of 2,400 and an average empirical formula of C_{145} H_{275} O_{20} NS. This principal sulfolipid is a 2,3,6,6′-tetraester of trehalose (91) which can be written as 2,3,6,6′-tetraacyl-α-α-trehalose 2′ sulfate. Tentative data regarding sulfolipids from *M. bovis* suggest that these "are not structured on a trehalose core sulfated in an equatorial secondary position" (92).

The property of acid-fastness is little understood. The idea that mycolic acids per se are responsible for acid-fastness seems ill founded (10, 216) and, in fact, *Mycobacterium* 1217

(kindly supplied to us for testing by G. Lanéelle and M. A. Lanéelle) is known to possess a full complement of mycolic acids, to produce waxy colonies, and to be nonacid-fast.

The guanine plus cytosine (GC) content for the *CMN* group offers a gradient from *Corynebacterium* (55 to 58%), to *Nocardia* (62 to 68%), to *Mycobacterium* (64 to 69%), (118, 290, 293, 306). The range of moles per cent GC found seems reasonable for three genera belonging to the same family.

The K antigens by which corynebacteria may be serologically identified are shown in Fig. 5 as protein located at the surface of the cell envelope. There is very little evidence as yet for the presence of such protein antigens in mycobacteria and in nocardias. However, fluorescent-antibody studies indicate that mycobacteria may be identified by their surface antigens (144). Further, there is evidence to suggest that a number of "tuberculin-active peptides" derived from the cell walls of *M. tuberculosis*, strain Aoyama B, may come from a superficial site in the cell envelope (15). It would appear that a fine-structure analysis of the mycobacterial and nocardial envelopes for use in an expansion of the system for the serological typing of these organisms could readily be accomplished with methods now available. There seems no doubt about the value of such an investigation.

Through the kindness of Bradley (143), Imaeda (132), Mankiewicz (189), and Redmond (255), we have assembled a collection of bacteriophages which when coupled with our own corynebacteriophages offers us a means of assessing the capacity of individual members of the *CMN* group to support the growth of some or none of these bacteriophages. As might have been expected, no phages were found which were capable of *multiplying* in more than one of the three genera. The use to which such a collection of phages may be put for clarifying certain taxonomic problems is illustrated by studies carried out with *C. rubrum* (56). We had suggested to Jose Antonio Serrano that *C. rubrum* was probably a *Nocardia* sp. on the basis of its general colonial and fermentative properties, its insensitivity to corynebacteriophages, and its sensitivity to nocardiophages. Chemical data to be found in his thesis (279) plus additional unpublished data of Lanéelle, Beaman, and Arden (this laboratory) indicate that the walls and lipids of *C. rubrum* are those of a nocardia, thus indicating the value of phage typing in examining unknowns such as this misnomer.

CORYNEBACTERIUM DIPHTHERIAE

C. diphtheriae is a collective designation for those members of the genus *Corynebacterium* which are capable of producing in human subjects a spreading, pseudomembranous growth on mucous membranes and in the skin, and sometimes causing serious obstruction in the larynx and trachea (72). [Infections rarely occur in animals (*see* **APPENDIX** and reference 93).] Such growth is an expression of the invasiveness or virulence of diphtheria bacilli (23, 116, 215) and is related to certain protein and lipoidal components associated with their surfaces (129, 153, 175).

Morphology, Ultrastructure, and Molecular Anatomy of Diphtheria Bacilli

The cells of a single strain of *C. diphtheriae* may range from slightly ovoid gram-positive units 0.5 to 1 μm in diameter to gram-variable rods 1.5 to 5 μm long (73). Certain mutants of the PW8 strain growing on blood agar form gram-variable filaments which extend over several oil immersion fields. Most diphtheria bacilli exhibit a uniform shape when growing at maximum rate in a medium satisfying all of their nutritional requirements. [The mean generation time obtained under optimal conditions of growth is very precise for each strain. Although all strains under such conditions are increasing logarithmically, not all strains reported as growing logarithmically are necessarily growing at maximal rate. Misunderstanding this point has led to confusion.]

Cell envelope. The dividing cells of strain $C7_s(-)^{tox^-}$ shown in Fig. 2 are typical of *C. diphtheriae* under optimal conditions of growth. The cells show a slight taper from the septal end (where they are still connected) toward the distal end. The layers of the cell envelope are discernible at the septal end and much less so at the distal end. These seeming laminations of the cell envelope are clearly evident in Fig. 2 in which the stages of ingrowth of the septum to final separation of the newly completed end walls are apparent. These pictures demonstrate what is in essence the "dividing with a snapping motion" commonly attributed to true corynebacteria (318). Some idea of the relation of the ultrastructure of these sections of *C. diphtheriae* to its molecular components may be obtained by comparing the pictures in Fig. 2, 6, and 7 with the drawing comprising Fig. 5.

Murein (peptidoglycan, muropeptide) and arabinogalactan. Kato, Strominger, and Kotani

(152), working with the PW8 strain, preparing its walls according to two procedures,[3] and using the L-3 enzyme preparation of Mori, Kato, Matsubara, and Kotani (205), showed that lysis of the walls of PW8 brought about by the L-3 preparation results from the action of a D-alanyl-*meso*-DAP endopeptidase which catalyzes the hydrolysis of interpeptide bridges connecting the peptide subunits of the murein. The major peptide units substituted on the acetyl muramic acid residues of the peptidoglycan they find to be the tetrapeptide L-Ala-D-Glu-*meso*-DAP-D-Ala and the tripeptide L-Ala-D-Glu-*meso*-DAP. Only a portion (perhaps 20%) of the tetrapeptide and tripeptide subunits were reported by these authors to be cross-linked through D-Ala-*meso*-DAP bridges. How the arabinogalactan (60, 120) is tied into the basic wall structure has not yet been determined. This polysaccharide is what Lautrop terms the O antigen (175), a group antigen common to the corynebacteria which cross-reacts with the arabinogalactans of mycobacteria and nocardias (57–60). The murein component is visible in Fig. 6 and 8. In the latter case (Fig. 8B), the cell is that of a *Corynebacterium* which under certain conditions makes an excessive amount of muropeptide (82). From the electron micrographs published by Lickfeld (181), it would appear that *intermedius* strains of *C. diphtheriae* are similarly unbalanced with regard to murein biosynthesis.

Lipids and protein of the outer envelope. In addition to peptidoglycan and arabinogalactan, there is considerable lipid associated with the corynebacterial envelope (87). Asselineau produced a masterful review of the literature pertaining to these lipids up to 1966 (10). Since many investigators of corynebacterial lipids did not separate the walls of the bacteria being analyzed from the contents of the cytoplasmic membranes, the molecular species they report often cannot be assigned anatomical sites. Kitaura et al. specifically called attention to lipids in the walls of *C. diphtheriae* in 1959 (160). As explained in the legend to Fig. 5, here we present the probable bacteriographic distribution of molecular components in a *CMN* bacterium from data available concerning corynebacteria, mycobacteria, and nocardias. In the case of *C. diphtheriae*, the major lipid components are corynemycolic and corynemycolenic acids, the cord factors or trehalose dimycolates, and phosphatides of mannose and inositol. It has long been thought that invasiveness of *C. diphtheriae* was in some way associated with lipoidal antigens of the cell surface (23). Alimova (5) was among the first investigators to localize the toxic activity now attributed in part or in toto to the cord factor (153) in ligroin extracts from the cell surface and to suggest that fatty acids or fatty acids in ester linkage with trehalose were responsible for the toxicity.

Cord factor of *C. diphtheriae*. The work of Ioneda, Lenz, and Pudles (133) and of Senn, Ioneda, Pudles, and Lederer (278) make it clear that the cord factor of *C. diphtheriae* is a mixture of diesters of α,α'-trehalose esterified in the 6,6′ position with any of several homologues of corynemycolic and corynemycolenic acids (278). Recently, Masahiko Kato showed that this corynebacterial cord factor is lethal for mice, that mouse mitochondria are disrupted by its toxic action, and that the resulting residual mitochondrial fragments are deficient both as to respiration and phosphorylation (153). Thus, the pharmacological action of α,α'-trehalose-dicorynemycolate appears to differ little from that of the cord factor of *M. tuberculosis* (34, 153). Cord factor activity has also been demonstrated in extracts from *C. ovis* (*pseudotuberculosis*), (45). It could well account for the results reported by O'Meara on a toxic fraction from washed *gravis* bacilli, even though his method of extracting the *gravis* strains studied by him did not involve the use of petroleum ether (223). The outermost layer of *C. diphtheriae* is not very firmly bound to the cell (*see* Fig. 2, and 6A and a later section in this paper dealing with iron phenotypes), and considerable amounts of free lipid may be removed by simply washing the cells. Although cord factor is undoubtedly important in the cellular response to *C. diphtheriae*, its mere presence cannot alone account for virulence or invasiveness because the PW8 strain from which it was originally isolated is a relatively noninvasive strain. Cord factor probably is a necessary adjunct of virulence, the K antigens of the surface being also required for invasiveness of diphtheria bacilli.

K (surface protein) antigens. The type-specific antigens of *C. diphtheriae* are heat-labile (175) proteins (316, 317) located at the cell surface (57). In keeping with the scheme used by Kauffman (155), Lautrop has called those protein

[3] The two procedures of Kato et al. are as follows. (i) Walls from sonically disrupted cells were separated by differential centrifugation and "purified" by treatment at 37 C for 2 hr with trypsin in 0.01 M phosphate buffer, washed with buffer followed by water, and freeze dried. (ii) Walls were delipidated by treatment with 40 volumes of ethyl alcohol–ethylether mixture (1:1, v/v) for 2 days at room temperature (procedure carried out three times) and, finally, the cell walls were extracted three or more times (for periods of 2 days each) in chloroform. (Note: for elucidating the relation of K antigens and dimycosides to the cell envelope, perhaps more delicate preparative methods must be employed.)

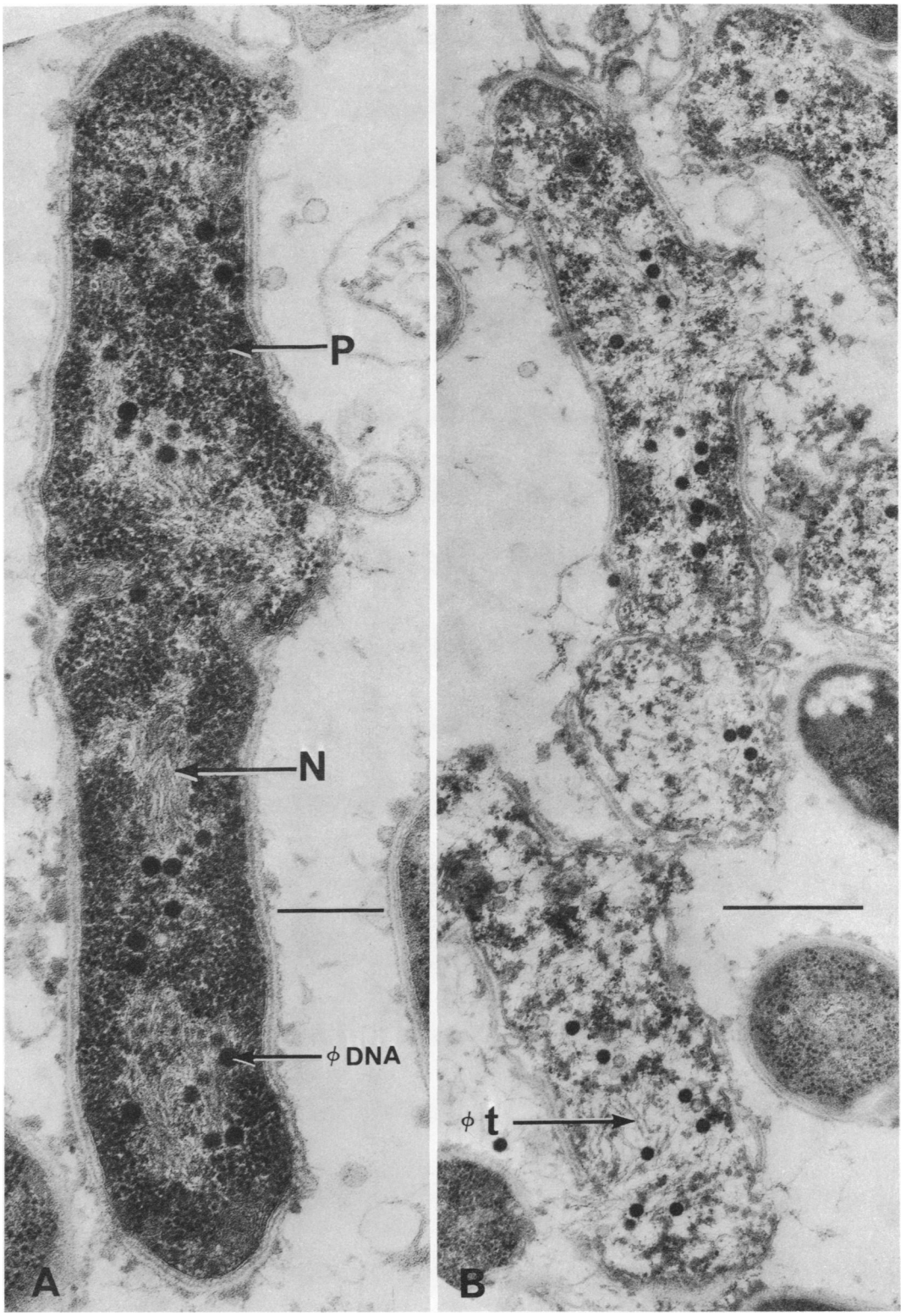
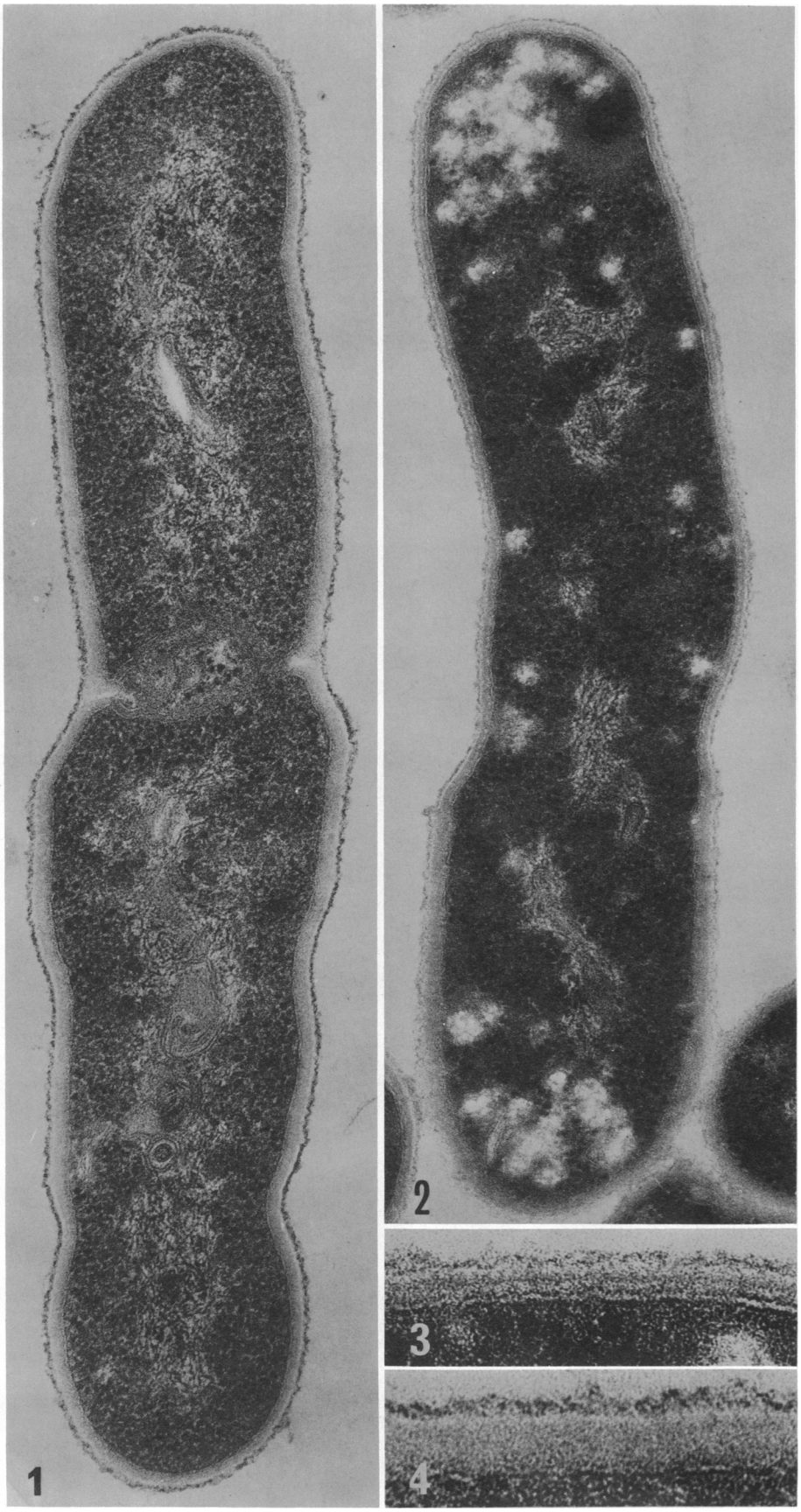


FIG. 6. *Cells of C. diphtheriae strain* $C7_s(-)^{tox^-}$ *fixed and sectioned from the periphery of a phage plaque (see Fig. 17). (A) Cell in which viral DNA synthesis is nearing completion. Note the distribution of viral and host DNA and the density of polysomes.* × *66,000, scale 0.25 μm. (B) Premature lysis of cells due, probably, to concentrations of lytic enzyme in their environment. Note that the cell envelope has been reduced to little more than the rigid layer (murein). Symbols: φ DNA, phage DNA; φ t, phage tails; P, polysomes; N, corynebacterial DNA.* × *43,000, scale 0.5 μm. From data of K. S. Kim, Sheila Heitner, and L. Barksdale.*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antigens responsible for the serological types of *C. diphtheriae* "K antigens." How the K antigens are bonded to the envelope remains to be discovered. Evidence for the role of these type-specific antigens in infection and immunity will be discussed under the section on **POPULATIONS OF CORYNEBACTERIA.** [For a listing of valuable contributions made to the serology of *C. diphtheriae* during the first two decades of this century, see Huang (128, 129).]

Receptors for corynebacteriophages. Although corynebacteriophages may be seen fixed to the surface of corynebacteria examined under the electron microscope, except for the studies of Groman and associates on a phage inhibitor released by *C. diphtheriae* in the presence of oleic acid (95–97) there has been no investigation of the nature of the receptors for these phages.

From the Nucleus to the Cytoplasmic Membrane (Cellular Inclusions)

The moles percent GC reported for DNA from a few strains of *C. diphtheriae* follow: 51.9 (178), 54.5 for strain Toulouse C 8384 (35), 54.4 for PW8 (148), 60 for Kareva no. 11 (148), and 54.5 (284). If one leaves out the first value, an early report in which the guanine content was low, the average per cent GC for this small sampling is 55.8. Percentages reported for related species are: *C. equi*, 58.5; *C. hofmanni*, 57; *C. kutscheri*, 58.5; *C. minutissimum*, 54.5; and *C. xerosis*, 55 (118). All values for the corynebacteria are below those of representatives of other arabinogalactan-containing genera such as, for example, *Nocardia corallina*, 62.3%, and *M. tuberculosis*, 64.9% (293). In thin sections of actively growing cells, the deoxyribonucleic acid (DNA) appears as a mass of electron-dense fibrils (*see* Fig. 10). In cells which are suffering from nutritional deficiency, the nuclear matter appears infused with fluffy electron-opaque areas (*compare* Fig. 2, 7, and 8).

Fatty material, lipoidal bodies and fat granules. Burdon (42), in a comprehensive study of the capacity of bacteria to be stained with Sudan Black B, noted that corynebacteria and mycobacteria contained lipid in "conspicuous amounts in nearly all mature cells." We find that in cells from slowly metabolizing populations large granules can be revealed with Sudan Black B. When a comparison is made between whole cells so stained with Sudan Black B and ultrathin sections of homologous cells stained with uranyl acetate, one is led to the conclusion that much of the lipid, localized as macrostructures in corynebacteria (and some mycobacteria), is associated with infoldings of the cytoplasmic membrane (mesosomes). In most mycobacteria, the lipoidal bodies appear larger, and in ultrathin sections they seem only rarely to be associated with the intracytoplasmic membrane system. These relationships are illustrated in Fig. 11.

When cells of strain $C7_s$, growing at maximal rate, are stained with Sudan Black B, there is a diffuse but definite fixation of the dye in the polar areas of the cell. It seems reasonable to assume that the lipid or phospholipid so demonstrated is responsible for the electron-opaque areas seen in ultrathin sections such as those comprising Fig. 2, 7, and 10.

Polyphosphate granules = metachromatic granules = volutin bodies. The inclusions in the corynebacterial cell which take on a purplish red to pink color after exposure to methylene blue or Toluidine Blue, do so by effecting an ordered polymeric arrangement of localized dye molecules so that there is a shift in the absorption peak (from 630 to ±540 nm in the case of Toluidine Blue), with the consequence that the blue dye is seen as pink (198). These refractile granules, first described by Babes (18) and Ernst (77), are composed of phosphate glass and belong to the same class of long-chain inorganic polyphosphates as Graham's salt (299–301). In addition to being revealed with dyes, they can be observed by staining with lead salts (302). They are found in all three genera of the *CMN* group. They are not discernible in corynebacteria growing at maximal rate (*see* Fig. 2), but are commonly found in cells whose growth is retarded, provided there is an adequate supply of phosphate in the medium (69, 70). Figure 9 shows polyphosphate granules of varying sizes in cells of the PW8 strain. Each granule has associated with it portions of membrane (mesosome). In sections of cells of *C. diphtheriae* growing at maximal rate, only small fragments of membrane (as opposed to large infoldings) and no polyphosphate granules are seen. Visible accumulations of each of these entities in *CMN* organisms seems to be an indication of a slowing of growth. That polyphosphate granules appear in old cells and are not found in actively dividing cells suggests that they are a store of pyrophos-

FIG. 7. *(1) Low-iron cell phenotype and (2) high-iron phenotype of* $PW8_r(P)^{toz+}$ *(× 80,000); (3) segment of cell envelope of cell grown in high-iron medium and (4) of cell grown in low-iron medium (× 237,000). Cells were fixed in glutaraldehyde and osmium and embedded in epon. From data of Kwang Shin Kim and L. Barksdale. (Reprinted from Essays in Microbiology with the permission of the Columbia University Press.)*

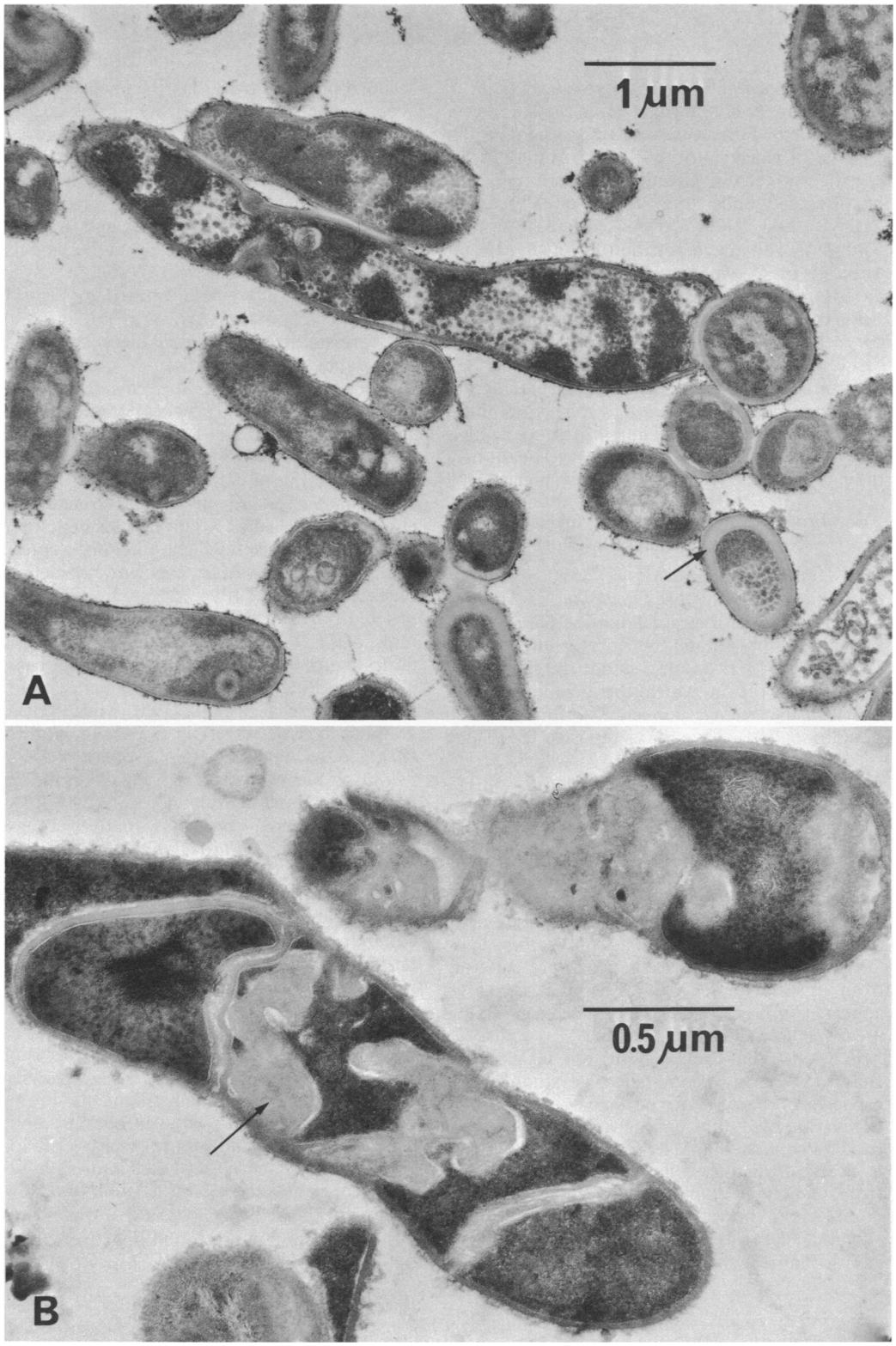

FIG. 8. *(A) Cells of PW8$_r$(P)$^{tox+}$ from a low-iron culture producing 18 μg of toxin protein/10^9 bacteria/ml. Arrows indicate exaggerated murein layer. × 20,500. Compare with Fig. 7 and 10. (B) Cells of Corynebacterium sp. 2628T60 growing under conditions which have led to unbalanced synthesis of the murein "layer." Arrows indicate exaggerated murein layer. × 46,500. For further information see text and reference 82.*

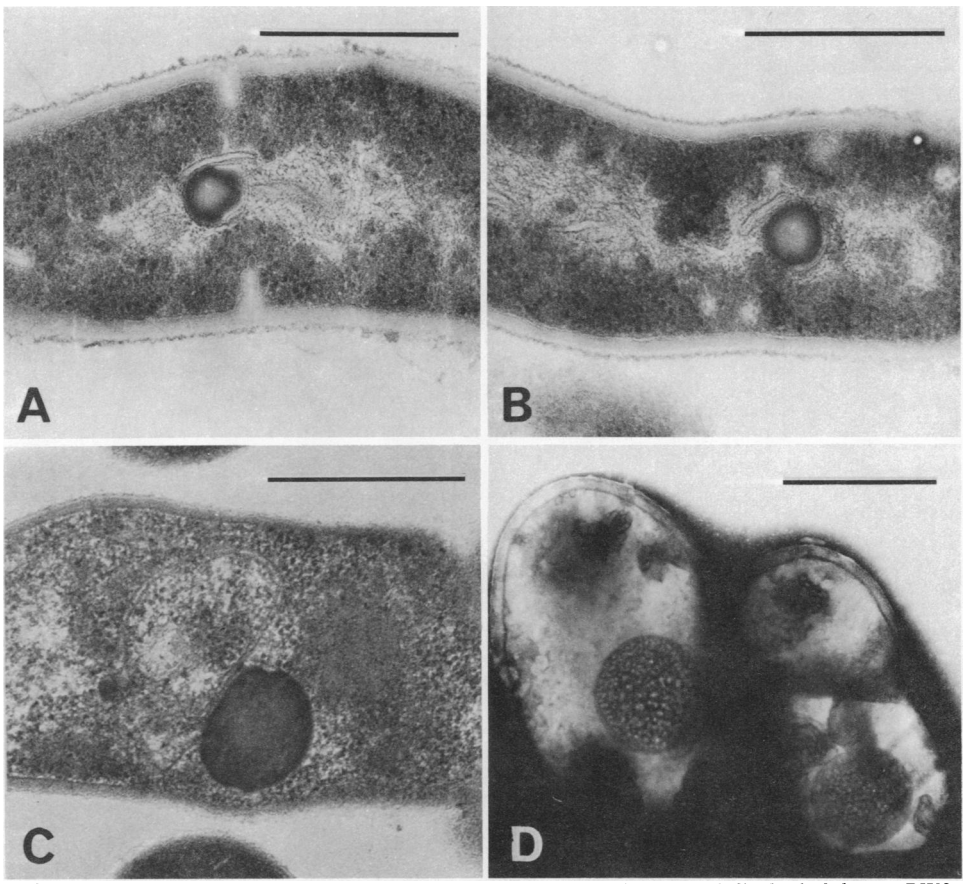

FIG. 9. *Metachromatic granules at various stages of development. (A, B, and C) C. diphtheriae PW8 strain, grown in low-iron medium, stained with uranyl acetate and lead citrate. × 52,000. (D). Corynebacterium sp. 2628T60 negatively stained with 2% ammonium molybdate. × 40,000; scale 0.5 μm. From data of Sheila Heitner and Kwang Shin Kim. For discussion of metachromatic granules in polyphosphate metabolism, see text; also note references 2, 68, and 163.*

phate for use at a time when the cell will be reactivated. The probable role of long-chain polyphosphates in the metabolism of true corynebacteria is discussed in the section on **Nutrition and Metabolism.**

Localization of tellurium. In 1900, Klett observed that a number of organisms including *C. diphtheriae* when growing in media containing salts of tellurium or selenium apparently reduced those salts to free metal (162). This observation led Conradi and Troch to devise for *C. diphtheriae* a semiselective medium containing potassium tellurite (54). In 1941, Harry Morton and Tom Anderson (208) found needle-like crystals in cells of *C. diphtheriae* and *C. xerosis* harvested from plates of tellurite agar. They suggested that "since the majority of the crystals are contained wholly within the cells, it is to be inferred that the tellurite or tellurous ion is able to diffuse through the cell wall and is there reduced to tellurium metal which is precipitated inside the cell." The black color of the cells and the needle-like crystals disappeared upon the addition of small amounts of bromine water. Tucker, Walper, Appleman, and Donahue added to this circumstantial evidence X-ray diffraction data compatible with the conclusion that crystals of tellurium do accumulate in *C. diphtheriae* (297).

Starch. Hehre, Carlson, and Neill in 1947 made the valuable observation that strains of *C. diphtheriae* growing in still culture in broth containing glucose-1-phosphate accumulated intracellularly an iodinophilic material (112). Carrier and McCleskey, examining a variety of "corynebacteria," showed that for certain true corynebacteria only glucose-1-phosphate serves as a substrate for starch formation (46). Arden,

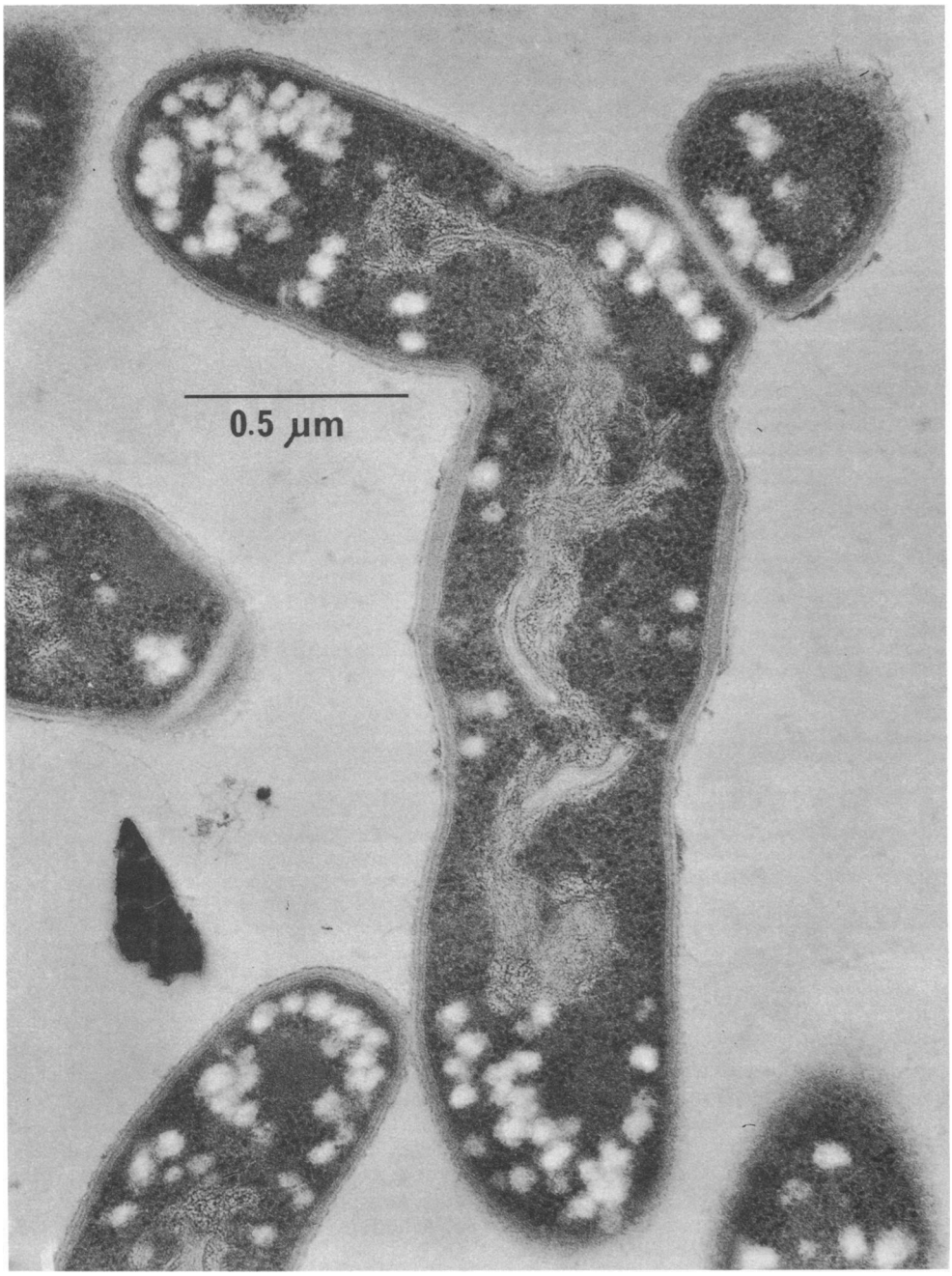

FIG. 10. *High-iron phenotype of PW8$_r$(P)$^{toz+}$ exhibiting rudimentary branching, a fully developed cell envelope, DNA and intracytoplasmic membrane, ribosomes, and electron-opaque areas.* × *78,000; scale 0.5 μm. From unpublished data of Kwang Shin Kim and L. Barksdale.*

in this laboratory, has found that all strains of *C. diphtheriae*, *C. ulcerans*, *C. ovis*, and *C. kutscheri* tested synthesize starch from glucose-1-phosphate and that aeration of the culture inhibits starch production (*unpublished data*). The glucan phosphorylase of these bacteria, then, seems associated with anaerobic metabolism.

Intracytoplasmic membrane = intracytoplasmic membrane system (125) = mesosome(s) (80, 268). Sections of corynebacterial cells such as $C7_s$-

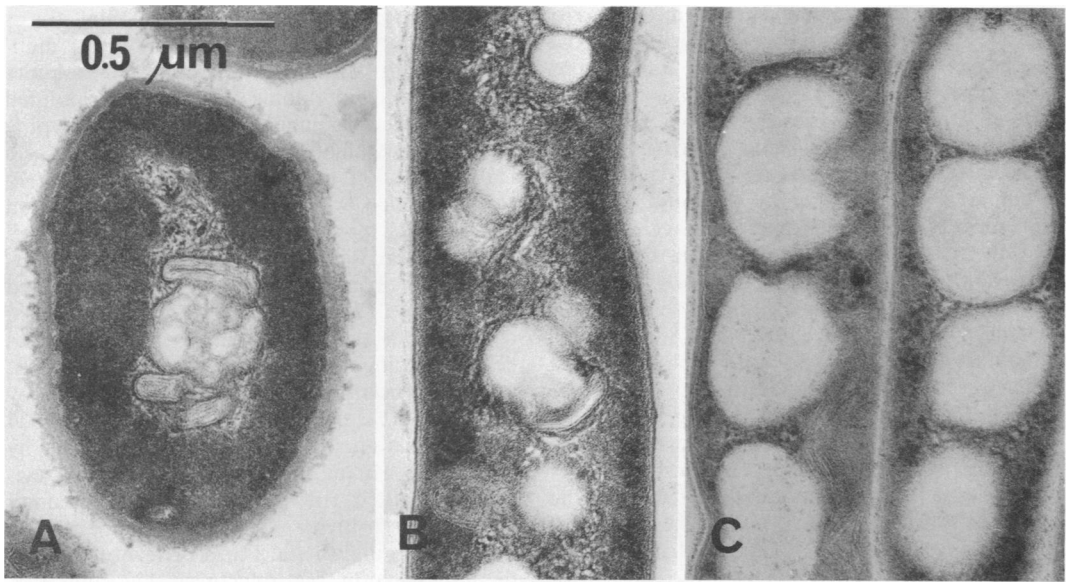

FIG. 11. *Areas associated with Sudan Black B-positive inclusions in (A) Corynebacterium sp. strain T60, (B) Mycobacterium aurum, (C) Mycobacterium sp., strain ICRC. × 65.000. Note degree of association of the intracytoplasmic membrane system with the electron-opaque areas in each case. From data of K. S. Kim and L. Barksdale.*

$(-)^{tox^-}$ growing at maximal rate (Fig. 2) show only traces of infoldings of the cytoplasmic membrane system. A variety of conditions which lead to slowing of growth lead to an accumulation of intracytoplasmic membrane (Fig. 7, 9). In our experience with corynebacteria, mycobacteria, nocardias, and propionibacteria, the accumulation of mesosomes or infoldings of the membrane system reaches its maximum in the resting or nondividing cells. Such cells have been used by Masahiko Yoneda and T. Uchida as a source of membranes for the study of the synthesis of diphtherial toxin. The site of toxin synthesis they consider to be the membrane (298).

The membrane system in *C. diphtheriae*, then, seems always to exhibit infoldings intimately associated with the nuclear material of the cell; these are minimal in cells growing at maximal rate and maximal in nondividing cells (*see also* 156). The extent to which a variety of enzymic activities (associated with membranes in other bacteria) are associated with the membrane system of *C. diphtheriae* has not been determined. A method for preparing corynebacterial protoplasts would be a great aid to studies of the corynebacterial membrane. Ziegler and Barksdale (*unpublished data*) found that growing the PW8 strain in the presence of 0.5 units of penicillin per ml, followed by exposure to lysozyme, yielded about 20% spherical cytoplasts. Mori, Kato, Matsubara, and Kotani reported the "protoplasting" of *C. diphtheriae* (PW8) using an enzyme (L3) from *Streptomyces* sp. (205). Lysis of the protoplasts occurred once they were pelleted and resuspended in distilled water. The time required for removal of the cell walls by the L3 enzyme, as measured by the disappearance of material agglutinable by anticell-wall antibody, was about 90 min.

POPULATIONS OF CORYNEBACTERIA

Colonial Morphology

It was pointed out under the discussion of the corynebacterial cell that in each case the superficial layer of the cell is a protein antigen, which Lautrop has termed the K antigen. Differences in surface antigens in corynebacteria, as with all bacteria, are reflected in the way the individual cells pile up to form a colony. Better-known examples of such colonial forms of bacteria are the smooth and rough pneumococci, smooth and rough *Escherichia coli*, smooth and rough *Shigella*, etc. Although smooth and rough *C. diphtheriae* had been recognized from about the turn of the century, it took the systematic work of the Leeds group under J. W. McLeod to obtain general recognition of the so-called colonial "types" *gravis*, *mitis*, and *intermedius* (196). By using the manifestation of toxaemic diphtheria as a means of selection, these workers established a correlation between the colonial appearance of the strain of *C. diphtheriae* re-

covered from a patient and the clinical severity of the infection; organisms producing smooth (S) colonies were associated with mild (*mitis*) infections, semirough (S-R) colonies were recovered from severe disease (*gravis*), and dwarf smooth colonies (*intermedius*) were associated with infections intermediate in severity. [As might be expected, mutations from SR to S and from SR to dwarf smooth have been reported (21, 262). For this discussion, the starch-fermenting ability of *gravis* strains is ignored. There are other peculiarities of these genotypes that need investigating (106, 107, 188).] It was, of course, a step forward for the diphtheriologist to have reemphasized the fact that *C. diphtheriae* is an epithet applied to an *assemblage* of corynebacteria, differing one from the other according to certain stable and easily recognizable properties; Dr. McLeod is certainly correct when he says that "the existence of these types has been so widely recognized and accepted that it cannot be considered to be any longer in doubt" (196). Unfortunately, this kind of analysis is today far too superficial. What is wanted is a way to tell one *gravis* strain from another, one *mitis* from a second *mitis*, etc. There is a variety of antigenic types of *C. diphtheriae* (100, 129, 175, 317), and some knowledge of them is essential for understanding precisely the occurrence of diphtheria (i) in persons immunized with toxoid and showing detectable levels of circulating antitoxin (134, 258), and (ii) in persons immunized by previous infection, as well as for appreciating fully such responses as the combined pseudoreaction to the Schick test. Infection with a particular antigenic type may or may not endow one with immunity to a second type. Any antigenic type, depending on its genetic constitution, may or may not produce diphtherial toxin. All toxins called diphtherial toxin appear to be immunochemically identical. An individual who survives infection with a toxinogenic strain (or who is immunized with toxoid) develops circulating antitoxin. When infected with a toxinogenic strain of a second antigenic type, such an individual will, of course, be immune to the toxin. The development of diphtheritic infections in individuals immune to toxin (i.e., in persons having serum levels of 0.1 to 0.5 au/ml) has often been reported by clinicians (31, 102) and has been confirmed in laboratory accidents (23) as well as in laboratory experiments (176). The importance of antibacterial immunity in the epidemiology of diphtheria, then, is now well established (128, 176, 188), though not generally appreciated (62).

Twenty years ago, Morton (207) indicated that in addition to the three colonial forms described by the Leeds workers there were diphtheria bacilli which grew as rough (R) colonies and (M) mucoid colonies. Morton also pointed out the difficulties inherent in a system of typing based on colonial morphology. (Morton also called attention to filterable forms of *C. diphtheriae*, which we on occasion have found in old "sterile" preparations of toxin.)

Serological types of C. diphtheriae. The heat-labile, protein antigens described by Wong and Tung (316, 317) and Huang (129) are localized on or about the surface of the corynebacterial cell, according to the work of Cummins (57), and have been redesignated as K antigens by Lautrop (175). Before the systematic serological characterization of these specific proteins by Wong and Huang, there was considerable evidence for their role in antibacterial immunity and hypersensitivity separate from antitoxic immunity in diphtheria. For a review of some of this information see references 128 and 176. Lautrop has defined the heat-stable (127 C for 2 hr) antigen as the O antigen of *C. diphtheriae*. He suggested that there is an O antigen common to all *C. diphtheriae* and that in smooth strains (*mitis*) there is an additional special O antigen. Presumably these O antigens represent modifications of the arabino-galactan portion of the wall [*see* Cummins (57) and the discussion of the *CMN* group herein]. The specific K antigens, the superficial protein layers, are quite distinct in the case of some corynebacterial strains but exhibit cross-reactions in the case of others. The PW8 strain belongs to a K antigen type designated as D-5 by Huang. Patients infected with strains of K(D-5) and treated with antitoxin, which had been produced in response to toxoid derived from the PW8 strain, showed a more rapid clearance of K(D-5) organisms from the throat than patients infected with heterologous strains such as, for example, K(D-6). Huang studied the appearance and disappearance of passively acquired agglutinins (*ex* antitoxin) as well as the agglutinins actively produced by the patients in response to the specific K type infecting them. It is worth noting that when a comparison was made between the number of deaths among patients infected with the type K(D-5), the strain homologous to PW8 and therefore related to the antitoxin used in treatment, and the number of deaths caused by other K types, the number was about the same in each group. Since most of the deaths were in infants and *were due to obstruction of the larynx and trachea*, it appears that the quantity of antibacterial γ-globulins contained in the antitoxin was insufficient to

exert an effect upon the pseudomembranous growth in these cases. [No internationally standardized system exists for the serological typing of corynebacteria. There is a real need for such a system to be used in conjunction with an internationally standardized system for the typing of these bacteria with bacteriophages.]

K antigens, adjuvant action, and the Schick test. The Schick test is a skin test designed to determine sensitivity to diphtherial toxin. As of this writing all diphtherial toxins, whether they are synthesized by *C. diphtheriae*, *C. diphtheriae* var. *ulcerans* or *C. ovis* (*pseudotuberculosis*) (see section on *tox* gene), are immunologically identical. The immune status of an individual, *with respect to diphtherial toxin*, may be determined through the use of a modified Schick test (102, 227, 230). Approximately 0.0006 μg of toxin protein, or one-fiftieth the minimal amount required to kill a guinea pig weighing 250 g, is injected into the skin of the forearm, the test site. An equal amount of heat-inactivated toxin is injected at a control site. Necrosis at the test site indicates a nonimmune state, whereas immunity, the presence of circulating antitoxin, is indicated by lack of reaction at either site. Immunity complicated by allergy to corynebacterial products results in a delayed inflammatory reaction at both sites. These latter reactions are to a large extent determined by the purity of the test material. It has already been pointed out that the PW8 strain, the organism used almost universally for the production of toxin, belongs to serological type K(D-5). The extent to which preparations of toxin and toxoid derived from the PW8 strain will detect hypersensitivity to diphtherial products will then depend on (i) the number of antigens they contain in addition to toxin and (ii) the extent to which these antigens are common to corynebacteria previously encountered by the subject being tested. [As pointed out by Pope (245), Moriyama (206), and others, even 5× crystallized toxin is not really pure.] Corynebacterial cells and subcellular components are good adjuvants as well as good antigens (166).

Phage typing corynebacteria. Saragea and Maximescu (269, 270) assembled a group of 24 corynebacteriophages with which they were able to distinguish 19 phage types of *C. diphtheriae*. Saragea and co-workers (270) found it possible to type some 75% of 12,000 strains of *C. diphtheriae* by employing their system of typing. It would be helpful to those who are concerned with problems of specifically identifying corynebacteria to have this system expanded along the following lines. The phage sensitivity of the types needs now be correlated with (i) serotype of the host strain, (ii) the restrictive versus permissive nature of the deoxyribonucleases of the host strains, (iii) the lysogenic immunity of the host strain, (iv) the repressor sensitivity, and (v) the serotype of the typing phage. Work towards such amplification deserves the support of the World Health Organization and such local organizations as the National Communicable Diseases Center, Atlanta, Ga. The way such a typing scheme can be used to detect fine differences among corynebacteria is illustrated in Table 1.

Nutrition and Metabolism

Nutrition. Howard Mueller indicated in his comprehensive review of the nutrition of *C. diphtheriae* that nutritionally nonexacting strains were outside the realm of his experience (210). We have searched without success for strains with simple growth requirements. Drew and Mueller (67) designed a completely defined medium which supports the growth and toxin production of the PW8 strain. It contains beta alanine, nicotinic acid, pimelic acid, cystine, glycine, valine, leucine, methionine, proline, glutamic acid, tryptophan, ammonium ion, and added salts of magnesium, copper, zinc, manganese, and iron. For everyday use, the casein hydrolysate medium of Mueller and Miller is a more practical one (211, 212). It is usually modified to contain added glutamate, pantothenate, and tryptophane (PGT) to take care of the needs of a variety of strains (107, 108). Certain groups of corynebacteria will not grow in this medium unless thiamine is also present. Of the strains currently used in our laboratory only the *intermedius* strains fail to grow in PGT medium plus thiamine. Hata et al. (110) were able to obtain reasonably good growth of *intermedius* strains on PGT medium to which had been added acetate and lactate.

Mueller and Cohen found that oleic acid much enhanced the size of colonies of *C. diphtheriae* growing on solid media (*see* 210).

For isolating and characterizing colonies of corynebacteria and for the commercial production of toxin (with rare exceptions), complex media are used.

Carbohydrate utilization. Diphtheria bacilli are facultatively aerobic organisms. It is well known that their fermentation of glucose leads to the creation for them of bacteriostatic and even bacteriocidal conditions in liquid media. Among the products of such fermentation are acetic, formic, and propionic acids, some lactic and some succinic acid, and traces of ethanol (292). However, when maltose is provided (or even galactose) as an energy source, bacteriostatic levels of acid are

TABLE 1. *Phage typing scheme for distinguishing closely related corynebacteria*

Host strain	Phage type of host strain	Typing phages[a]									
		Z·603	Z^v·603	Z·C7	Z^v·C7	Z·21	Z^v·21	β·603	$β^v$·603	β·C7	$β^v$·C7
C7 (−)	1	−	−	+	+	−	−	−	−	+	+
C7 (Z)	2	−	−	−	+	−	−	−	−	+	+
C7 (β)	3	−	−	+	+	−	−	−	−	−	+
C7 (Z, β)	4	−	−	−	+	−	−	−	−	−	+
603	5	+	+	+	+	+	+	+	+	+	+
603 (Z)	6	−	+	−	+	−	+	+	+	+	+
603 (β)	7	+	+	+	+	+	+	−	+	−	+
603 (Z, β)	8	−	+	−	+	−	+	−	+	−	+
21	9	−	−	−	−	+	+	−	−	−	−
21 (Z)	10	−	−	−	−	−	+	−	−	−	−
C7/β	11	−	−	+	+	−	−	−	−	−	−
C7/Z	12	−	−	−	−	−	−	−	−	+	+
603/β	13	+	+	+	+	+	+	−	−	−	−
603/Z	14	−	−	−	−	−	−	+	+	+	+
21/Z	15	−	−	−	−	−	−	−	−	−	−

[a] Abbreviations: Z·603, Z phage produced in *C. ulcerans*, strain 603; Z^v·603, Z^v, virulent mutant of Z phage, produced in strain 603; Z·C7, Z phage modified in *C. diphtheriae*, strain C7; 603 $(β)^{tox+}$, strain 603 carrying prophage β and therefore immune to lysis by β phage; 603/β, a mutant of strain 603 lacking receptors for phage β. The discrimination possible in the system shown in this table depends upon the properties: host range, bar mutation, lysogenic immunity, and restriction and modification. The unique restricting and modifying hosts are C7 and 21. Note that each host strain has an individual pattern of sensitivity to the phages employed and therefore can itself be specifically typed. (See discussion of phage typing in text.) Symbols: +, forms plaques; −, does not form plaques (Arden, Pollice, and Barksdale, *unpublished data*).

not produced. Whether the temperance imposed by slowly deriving glucose from maltose (or transforming galactose) is responsible for the beneficial effects of these sugars is a matter for conjecture. In the metabolism of glucose by *C. diphtheriae*, there is evidence for the operation of both the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate pathway (PP = Entner-Doudoroff). Hulanicka (131) was the first to find evidence for the presence in *C. diphtheriae* (PW8 strain) of sedoheptulose and its transformation to hexose. He took this as evidence for the presence of a related phosphopentose isomerase, epimerase, transketolase, and transaldolase and for a pentose phosphate pathway in this bacterium. This is in agreement with the work of Zagallo and Wang (324) who, employing radiorespirometric techniques and using as carbon sources glucoses or gluconates labeled in different carbon atoms (*1*-^{14}C, *2*-^{14}C, *3*-^{14}C, *3*, *4*-^{14}C, and *6*-^{14}C), concluded that in the utilization of glucose and gluconate by *C. xerosis* the pentose pathway was of major importance. These investigators found glucose utilization by *C. equi* to involve about equally the EMP and the PP.

Jannes, Saris, and Jannes (137) have begun a study of reduced nicotinamide adenine dinucleotide (NADH) oxidoreductase-linked enzymes in strain CN2000 grown under two conditions: (i) aerobic in which the bacteria grew as an undisturbed pellicle and (ii) anaerobic in which the bacteria grew as bottom growth obtained by periodically disturbing the flasks so that the pellicles fell to the bottom. The enzymes looked at were L-iditol dehydrogenase, lactate dehydrogenase, malate dehydrogenase, and succinate dehydrogenase. From the standpoint of those interested in electron transport in *C. diphtheriae*, the finding that the malate enzyme, unlike the others, was made in large amounts by bottom-grown cells and in small amounts by cells growing on the surface should be of considerable interest.

Edwards has given evidence for the presence of a powerful lactic dehydrogenase in certain strains of *C. diphtheriae* growing in submerged culture in a complex medium containing 0.5% (v/v) glacial acetic acid, 2.4% (w/v) maltose, and appreciable amounts of lactic acid derived from the beef used in the preparation of the medium (74). Within 6 hr, at a time when the viable count ranged between 10^8 and 10^9 organisms per ml, 1 mg of lactic acid per ml had disappeared from the medium. Pyruvic acid levels reached a peak at about 6 hr. As the number of bacteria increased from the 12th to the 48th hr (from 10^9 to 10^{10} viable organisms/ml) the pyruvate completely disappeared.

Metabolism and long-chain polyphosphates. Under the section on cellular inclusions, it was mentioned that granules of long-chain polyphosphate found in the cells of members of the *CMN* group might serve as phosphate stores. Sall, Mudd, and Davis (267) studied the appearance and disappearance of polyphosphate granules in resting cells of *C. diphtheriae* which were incubated (i) in the presence of glucose and (ii) in the presence of malate. In the cells incubated with glucose, there was a gradual loss of granules without any appearance of phosphate outside the cells. In the cells incubated with malate, there was a steady increase in the number of granules. Distribution studies of the phosphorous components of the polyphosphate granule-containing cells, before and after incubation with glucose or with malate, indicated that in the presence of glucose ribonucleic acid (RNA) phosphorus increased at the expense of polyphosphate, whereas in the presence of malate, polyphosphate increased at the expense of the RNA phosphorus (267). M. Szymona and O. Szymona have shown that the enzyme preparations from the PW8 strain of *C. diphtheriae* can phosphorylate glucose using either adenosine triphosphate (ATP) or inorganic polyphosphate (288). S. R. Kornberg found in both $C7_s(-)^{tox^-}$ and $C7_s(\beta)^{tox^+}$ an enzyme which converts polyphosphate and adenosine diphosphate (ADP) to ATP (164). Jean-Pierre Ebel has for sometime been concerned with these polyphosphates and their role in the economy of bacterial cells. Dirheimer and he have found in *C. xerosis* (i) a polyphosphate-glucose and -glucosamine phosphotransferase and (ii) a polyphosphate-adenylate phosphotransferase. *The former* enzyme catalyzes the transfer of phosphate groups to the hydroxyl substituent at C6 in both glucose and glucosamine. The authors offer suitable evidence to show that the phosphate groups are moved directly from long-chain inorganic polyphosphate to hexose or hexosamine; i.e., there is no involvement of an intermediate formation of ATP (63–65). *The latter* enzyme catalyzes the phosphorylation of adenosine-5′-monophosphate in the presence of long-chain inorganic polyphosphate (Graham's salt). It is highly specific with regard to adenosine-5′-monophosphate (i.e., with regard to the 5′ position); adenosine-3′-monophosphate is not phosphorylated by it, nor are the other 5′ nucleoside monophosphates GMP, UMP, CMP, and IMP. It also is specific for inorganic polyphosphate (Graham's salt); orthophosphate, pyrophosphate, and trimetaphosphate do not serve as sources for phosphorylation (65). In addition to these phosphorylases, there have been described from *C. xerosis* phosphatases which degrade polyphosphate as well as an ATP-dependent phosphopolymerase (213, 214). Thus there is evidence for a role of polyphosphate *in the active metabolism* of the cell as well as *in the storage* of phosphate. Dirheimer and Ebel have proposed a cyclical scheme for the role of the enzymes (so far discovered in the *CMN* group and mentioned here) in the phosphate-related cellular processes. (i) ATP accumulated through oxidative phosphorylation may contribute to the polyphosphate stores via polyphosphate synthetase; (ii) such polyphosphate can serve as a source for the phosphorylation of ADP via polyphosphate-adenylate-phosphotransferase, or (iii) for the phosphorylation of glucose via polyphosphate-glucose-phosphotransferase. (iv) Adenylatekinase would take care of the interrelated interconversions of ATP ↔ ADP ↔ AMP and shortages of orthophosphate would be met by the interaction of polyphosphatase with polyphosphate stores.

Since phosphate derived from polyphosphate may go into ATP, such phosphate becomes involved in the metabolism of lipids and nucleic acids as shown in Fig. 12 (from Ebel reference 71). As early as 1950, Belozerski surmised, from very little data, that in *C. diphtheriae* polyphosphate was intimately associated with RNA, in the case of actively growing cells (30). His subsequent work with *Aspergillus niger* indicates that in actively growing mycelia supplied with Pi as P^{32}, most of the radioactivity is found in the acid-insoluble polyphosphate which is in some way "bound to RNA" (170). Belozerski's findings coupled with the data of Dirheimer and Ebel (63–65) suggest that in actively metabolizing cells there are probably many nidi of polyphosphate intimately associated with RNA. The location of the most intense biosynthetic activity in corynebacterial cells growing at maximal rate is probably at loci where new extensions of the cell are being made. These are the areas marked by electron-opaque spots in Fig. 2, 7, and 10. As pointed out elsewhere, such areas of whole cells show a diffuse but polar fixation of Sudan Black B. In all

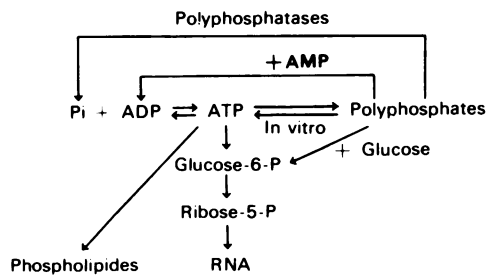

FIG. 12. *Use of polyphosphate by members of the CMN group [after Jean-Pierre Ebel (71)].*

probability, RNA-polyphosphate is prevalent in the same area.

Iron and *C. diphtheriae*. G. L. Eichhorn has called iron "the most versatile of all biochemically active metals" because it is an integral part of a variety of biological molecules essential to a number of biosynthetic processes (76). Most of the information available on the role of iron in bacterial metabolism has been gained incidental to research on iron-containing pigments and enzymes, iron-binding molecules, or the results of investigations of iron deficiency. All such studies are best carried out with media containing controlled levels of iron. Rendering media suitably iron free can be accomplished by the method of Mueller in which iron is removed by coprecipitation with calcium phosphate (211) or by complexing with hydroxyquinoline as described by Waring and Werkman (304) or, less specifically, by exploiting the iron-binding capacity of bacterial cells (322) or the iron-sequestering ability of cell walls and cytoplasm of yeasts (6).

In the matter of iron metabolism, the first step for the bacterial cell is that of inward transport. Two general means of iron transport have been assumed for bacteria: (i) by "simple diffusion" and (ii) by means of special iron-chelating molecules synthesized by the bacterium. Some bacteria, unable to synthesize iron-binding molecules, exhibit an absolute requirement for such molecules. Examples of bacteria requiring iron-chelating factors for growth include *Arthrobacter terregens* (183, 219) and *M. johnei* (282). Many bacteria synthesize iron-gathering molecules, especially under conditions of iron deficiency. The well-known *terregens factor* produced by *A. pascens* (43) and *nocardamin* secreted by certain species of *Nocardia* (157) contain hydroxamate groups, —CON(OH)—, capable of forming coordination complexes with ferric ions. (Concerning ferrioxamines produced by actinomycetes, *see* reference 247.) The gram-negative species, *E. coli* and *Salmonella typhimurium* may utilize for chelation of iron the phenolate groups in such compounds as 2,3-dihydroxybenzoylglycine [Ferrichrome, (219)], 2,3-dihydroxybenzoylserine (222, 303) and the cyclic polyester, enterobactin, which consists of three residues of 2,3-dihydroxybenzoylserine (242). The most elaborate of the low-molecular-weight, iron-chelating compounds to date appear to be the mycobactins, which contain both hydroxamate and phenolate groups in the same molecule. A variety of mycobactins is synthesized by the mycobacterial species *M. aurum*, *M. fortuitum*, *M. kansasii*, *M. marinum*, *M. phlei*, *M. smegmatis*, *M. thermoresistible*, and *M. tuberculosis*. The optimal concentration of mycobactin for the growth of *M. johnei* varies with the different mycobactins. For example, concentrations of mycobactins M or N, above the optimum, actually depress growth. Further, there is a mutual antagonism between certain mycobactins when added together to cultures of the Johne Bacillus (282). The condition which leads to the synthesis of iron-chelating compounds appears to be simply one of iron deficiency. Once the chelated iron has entered the cell, it must find its way either into heme or into nonheme-containing compounds. The mechanism of such relocation is not known.

Although two members of the *CMN* group, *Mycobacterium* and *Nocardia*, produce well-characterized iron-chelating compounds, there appears to have been no report of such specialized molecules being produced by *Corynebacterium*. Perhaps they have not been sought. [Hori (126) examined the iron-chelating capacity of diphtheria toxin using $FeCl_2$ and $FeCl_3$. He found that ferrous ion is the form always associated with toxin even when ferric ion is the form which is added to the toxin. At iron-to-toxin ratios of 17:1, iron precipitated quantitatively with toxin. Prior addition of α,α'-dipyridyl, *o*-phenanthroline, ascorbic acid, or ethylenediaminetetraacetate prevented such precipitation.]

Ferrous ion, ferric ion, and the growth and ultrastructure of *C. diphtheriae*. In the production of diphtherial toxin for the making of toxoid, there was from the outset the problem of how to improve yields of toxin. Park met this problem by finding a strain, the Park Williams 8 strain, which produces 10 to 20 times as much toxin as the average diphtheria bacillus. [The strain was isolated from a patient (233).] Mueller, in working out the nutritional requirements of the PW8 strain, devised a mixture of trace metals which is today an integral part of both complex and defined media employed for toxin production (*see* section on **Nutrition**). Among the metals of importance for the growth of *C. diphtheriae* was, of course, iron. Iron was essential for growth, and maximal growth was necessary for maximal yields of toxin. In 1931 Locke and Main (184) and in 1932 Pope (243) reported that iron in large concentrations had an inhibitory effect upon toxin production. These workers were employing complex media. In 1936 Pappenheimer and Johnson, working with a defined medium, independently discovered the inhibitory effect of iron upon toxin production (228). These independent observations differed in at least one important respect. Pope worked with a complex medium containing chelating agents in which the effect of iron upon toxin production was not so dramatic. Pappenheimer used a defined medium in which far less iron seemed needed to depress the yields of toxin. When an excess of calcium ion and phosphate is present in a defined

medium, the inhibitory effect of iron is far less dramatic. With this added information, it is apparent that chelated iron does not inhibit toxin production to the same degree that free iron does. [Details concerning the chelation of iron by peptone and beef extract can be found in a paper by Mitchiteru Hori (126).]

Edwards and Seamer examined the effect of ferrous and ferric iron on growth and toxin production. They found that the ferrous and ferric forms were taken up equally well by organisms which on a weight basis produced similar final titers of toxin. However, ferrous iron appeared to exert its effect on toxin synthesis much earlier in the growth period than did ferric iron (*see* Table 2). Thus, these authors suggested that "perhaps only ferrous iron is inhibitory and that ferric iron requires to be converted to the ferrous form before exerting its effect" (75).

Yoneda examined the iron-binding capacity of diphtheria bacilli (322) and found them to readily take up iron. Bell (22) observed growth curves of populations of three strains of diphtheria bacilli initially grown in levels of iron suitable for division at maximal rate and then washed and subcultured them in the presence of 0.15 μg of added iron per ml and in the absence of added iron. Although both groups of cells exhibited a log phase of growth, only the cells which were growing in 0.15 μg of iron per ml grew at the maximal rate of division established for them. When he cultivated three populations of diphtheria bacilli in the presence of 1.0, 0.075, and 0.0 μg of added iron per ml and then twice washed them in deferrated medium, resuspended them in deferrated medium and observed their growth, the amount of growth observed reflected the amount of iron reserves bound by the cells of each population. In Fig. 13, it is apparent that even the most iron-starved population is capable of an increase in optical density equivalent to two divisions, albeit at much slower than normal rate. When these cells are examined under the light microscope, they are seen to be much longer than normal cells. They are not dividing but undergoing much elongation. This failure of or extreme delay in division appears to be the hallmark of the iron-starved cell. Alouf and Barksdale (*unpublished data*) found low-iron cells to be twice as fragile as high-iron cells to sonic vibration and to disruption with glass beads. This was true for both toxinogenic and nontoxinogenic strains. Recently, Kim and Lanéelle (*unpublished data*) found the ratio of free lipids to bound lipids to be significantly different for cells grown in high iron, i.e., higher, as compared with cells deprived of iron. Kim has shown that high-iron cells exhibit a different ultrastructure from low-iron cells, as can be seen by examining Fig. 7, 8, and 10 from which it is apparent that the high-iron cells have more layers in the cell envelope and and contain numerous electron-opaque areas. The low-iron cells have fewer or no electron-opaque areas and exhibit a much simpler cell envelope.

The prolonged division time seen in low-iron cells is an overall reflection of the state of iron insufficiency. Even in cells which are dividing at almost normal rate, such processes as phage multiplication are slowed down. The slow down is directly related to the extent to which the stores of iron of the cells are limited. In Fig. 14, it is clear that iron limitation has a delaying effect on the latent period of phage infection in $C7_s(-)^{tox^-}$.

Sickles and O'Leary added still another property by which high-iron and low-iron cells may be distinguished (281). They compared the production of extracellular proteins by $C7_s(-)^{tox^-}$ and $C7_s(\beta)^{tox^+}$ under conditions of 600 and 56.4 μg of Fe/liter. The high-iron cells yielded negligible amounts of protein, whereas the low-iron-grown $C7_s(-)^{tox^-}$ cells produced 27.8 mg of protein and $C7_s(\beta)^{tox^+}$ produced 28.8 mg of protein (dry weight). The incorporation of L-methionine-*methyl*-^{14}C into these proteins was 26,000 counts per min per mg in the case of $C7_s(-)^{tox^-}$ and 30,000 in the case of $C7_s(\beta)^{tox^+}$.

Heme iron (Fe_h), nonheme iron (Fe_{nh}) and the

TABLE 2. *Toxin production by PW8 strain CN2000 growing in media containing different concentrations of ferrous and ferric ion*[a]

Expt	Type of iron	Amt of iron (μg/ml)	Amt of toxin[b] at hour							
			6	12	18	24	30	36	42	48
A	Ferrous	1.34	0	0	0	3	7	27	46	54
B	Ferric	1.22	0	1	4	12	23	35	55	68
C	Ferrous	0.55	0	0	8	55	98	132	164	176
D	Ferric	0.85	0	4	12	31	58	74	92	108

[a] From Edwards and Seamer (75).
[b] Expressed as flocculation units (Lf) per milliliter.

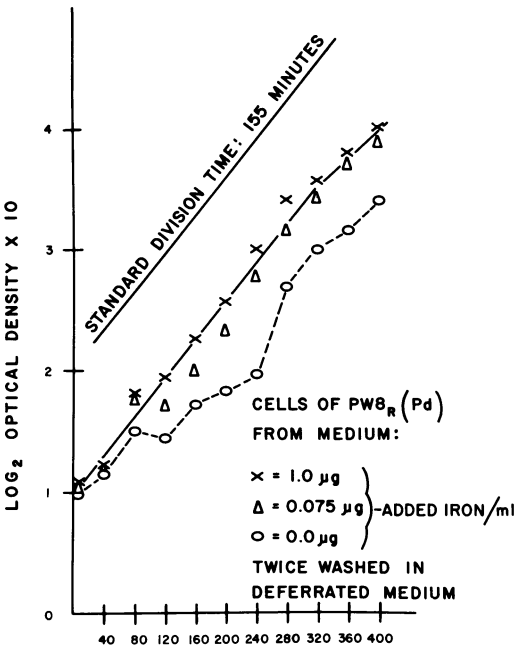

FIG. 13. *Effect of cell-bound iron upon growth of the* $PW8_r(P)^{tox^+}$ *strain in deferrated medium (from data of Ernest Bell and L. Barksdale). For the interpretation of increase in optical density under these conditions, see text. Reprinted from Essays in Microbiology, Columbia University Press.*

iron phenotypes. It is clear from the foregoing that the low-iron phenotypes appear to be more fragile than their high-iron counterparts, to undergo lengthening without dividing, to have a more simple wall structure, to possess a different ratio of bound to free lipids, to have their biosynthetic capacities slowed, and to secrete more protein into the medium (regardless of the nature of that protein). Righelato and van Hemert (261) recently examined the synthesis of toxin by batch- and chemostat-grown cultures of the PW8 strain, CN2000, and Righelato (260) looked into the matter of the partition of iron into Fe_h and Fe_{nh} in cells grown in 97 μg atoms/liter (excess iron) and in cells grown in 7 μg atoms/liter (low iron). The high-iron cells (97 μg atoms/liter) contained per gram of bacterial protein 15 μg atoms of Fe_{nh} and 0.58 μmole of Fe_h, and were extracellularly associated with 0.38 μmole of coproporphyrin and no toxin. The low-iron cells (7 μg atoms/liter) contained per gram of bacterial protein 1.03 μg atoms of Fe_{nh} and 0.16 μmole of Fe_h, and were extracellularly associated with 1.67 μmoles of coproporphyrin and 1.51 μmoles of diphtherial toxin. The amounts of Fe_h and Fe_{nh} found in the low-iron cells (measured as per cent of that found in the high-iron cells and calculated as microgram atoms of iron per gram of bacterial protein) were: (i) broken bacteria, Fe_{nh} = 6.7, Fe_h = 28, catalase from Fe_h = 10; (ii) soluble fraction, Fe_{nh} = 62, Fe_h ----, catalase from Fe_h = 10. The addition of iron to low-iron cultures resulted in a spurt of succinate dehydrogenase activity, suggesting to the authors that the succinate enzyme in low-iron cultures might itself be deficient in iron. The NADH oxidase activities were similar in the low- and high-iron phenotypes. Here, then, at the molecular level, are differences in the way iron is partitioned as Fe_h and Fe_{nh} in the iron phenotypes of *C. diphtheriae*.

Throughout nature the differences between the anaemic and the haemic are indeed profound. Such is to be expected, for the biological activities with which iron is associated are quite varied and most of them are vital to the living cell. In summary, it seems worthwhile to mention a few areas of bacterial metabolism affected by Fe_{nh} and therefore affected by iron deficiency. Fe_{nh} has

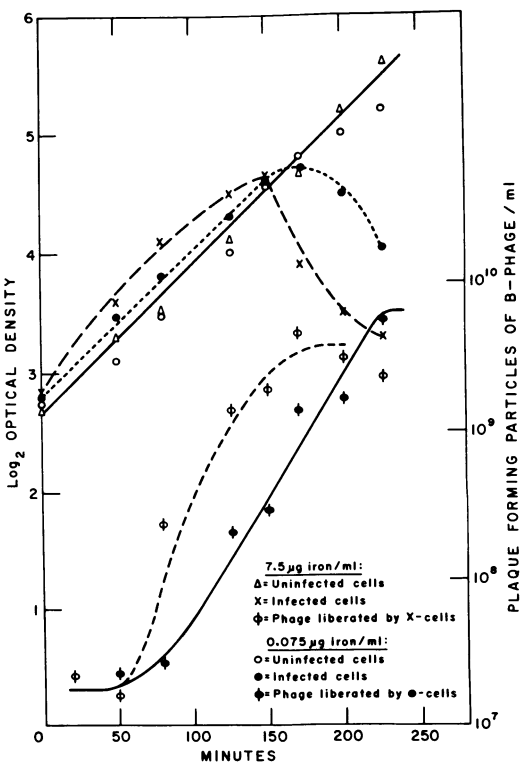

FIG. 14. *Effect of slight limitations of iron (see growth curves) on the multiplication of phage* β^c *in* $C7_s(-)^{tox^-}$. *From data of Martinello and Garmise in this laboratory.*

been shown to be involved in the respiratory pathways of *M. phlei* (252). Fe_{nh} appears to be a cofactor for a ribonucleotide reductase of *E. coli* (39). The 4-aminoimidazole hydrolase described by Rabinowitz and Pricer from *Clostridium cylindrosporum* is dependent on Fe^{2+} or other divalent cations for activity (251). The dihydroorotic dehydrogenase of *Zymobacterium oroticum* contains iron and therefore is an Fe_{nh} enzyme (201). It has long been known that iron, as well as pyridoxal phosphate, is required for the histidine decarboxylase activity of *Lactobacillus* sp., strain 30a (99). Recently, Harris considered some aspects of the effect of iron deficiency on nucleotide levels in *M. smegmatis* (105). He attempted to explain the low levels of uracil monophosphate found in such cells on the basis of the requirement of dihydroorotic dehydrogenase for Fe^{2+}. Winder and Coughlan found that a nucleotide triphosphate-dependent DNA breakdown system in *M. smegmatis* is substantially increased under conditions of iron deficiency (314). In the case of certain species of transfer RNA (tRNA) in *E. coli* (those which recognize some of the codons beginning with U) it appears, for example, that Fe^{3+} is involved at some stage in the enzymic modification of the C_2 position of the adenine adjacent to the 3′ end of the anticodon, $tRNA^{Tyr}$. The $tRNA^{Tyr}$ which is made under conditions of iron deficiency lacks an oligonucleotide found in $tRNA^{Tyr}$ made under conditions of iron sufficiency (264). So, there are many, many events which can be correlated with iron deficiency. If ferredoxins, now known to occur in aerobic *Azotobacter vinelandii* (280), are subsequently found among the *CMN* group, then iron deficiency in these organisms will inevitably lead to problems concerned with iron-containing compounds involved in electron transport on the hydrogen-side (just now reviewed by Buchanan and Arnon, reference 41) as well as on the oxygen-side of the dinucleotides, nicotinamide adenine dinucleotide phosphate and nicotinamide adenine dinucleotide (NAD). (Research concerned with the effects of iron-deficiency upon the heme-containing electron carriers of *C. diphtheriae* will be considered under the heading **Cytochromes.**)

Enzymes, Pigments, and Products of Special Interest

Bacteriocins. Thibaut and Fredericq (294) described bacteriocins active on and produced by corynebacteria. Such materials have been termed corynecins by Krylova (168) and Tashpulatova (291). Aside from their growth-inhibitory properties, these substances remain to be characterized.

Catalase. Catalase activity is a useful taxonomic property of true corynebacteria. It would be more useful were there easy ways of distinguishing the catalases of the different groups now comprising the genus. Robinson has shown that corynebacterial catalases (and esterases and peroxidases in the case of the plant "corynebacteria") can easily be separated by starch gel electrophoresis as well as by electrophoresis in polyacrylamide gel (263). Long ago, Herbert demonstrated a general approach to the crystallization of catalase from a gram-positive bacterium (113). A valuable contribution to the study of corynebacteria could come from a comparative study of their catalases. Edwards has found that the catalase content of diphtheria bacilli growing in submerged culture is a reflection of the growth curve (73) as shown in Fig. 23B.

"Cystinase," H_2S production. A medium widely used today for attempting the differentiation of *C. diphtheriae* from "diphtheroids" exploits the fact that strains of *C. diphtheriae* in the presence of thiosulfate are able to produce H_2S from cystine or cysteine. When H_2S is formed in the presence of K_2TeO_3, tellurides are produced around the colonies in the form of dark brown halos. The cystine-tellurite medium was originally described by Tinsdale (295). A recent discussion of its use is to be found in a paper by Porten (246) who used a modified Tinsdale medium for distinguishing "toxigenic strains from diphtheroids." She concludes that her statistics show "that 20% (32/159) of the cultures produced halos: of these halo producers 81% (26/32) were . . . toxigenic strains of *C. diphtheriae*. The remaining 19% (6/32) can be regarded as nonspecific reactions by diphtheroids. . . ." By Miss Porten's definition, $C7_s(-)^{tox-}$ is a "diphtheroid" and $C7_s(\beta)^{tox+}$ (*see* Fig. 1) is not. Both produce halos on Tinsdale's medium and, of course, both are *C. diphtheriae*. "Cystinase" production appears to be a useful genetic marker even though it is not an indicator of toxinogeny.

Cytochromes. Cytochromes *a*, *b*, and *c* were first reported in *C. diphtheriae* and *M. tuberculosis* by Hidetake Yaoi and Hiroshi Tamiya in 1928 (321). Pappenheimer (225) and Pappenheimer and Hendee (231) became interested in these respiratory pigments in relation to the iron metabolism of *C. diphtheriae*, and Pappenheimer, Howland, and Miller (232) examined the cytochromes of several strains of *C. diphtheriae* in relation to their division times, oxygen uptake, and catalase activity. They found the strains $C7_s(-)^{tox-}$ and $C7_s(\beta)^{tox+}$ each to contain a full complement of cytochromes (b_{564}, c_{552}, a_{600}) and, from the standpoint of respiration, to be es-

sentially identical. Interesting among the studies they reported was the effect of 2,8-bis-dimethylaminoacridine (acridine orange) on the $C7_s$-$(\beta)^{tox+}$ strain; after incubation for 15 hr in the presence of 15 μg of acridine orange per ml, the viable count dropped "from 10^6 to 1.5×10^3." All of the survivors were small-colony types, all grew at a slow rate (less than half the growth rate of the ancestral strain), all required less iron than their progenitor for growth, all excreted large amounts of porphyrin into the medium, and all had become unable to synthesize diphtherial toxin. One of these survivors, C7SC, was examined for its cytochrome content by determining its reduced minus oxidized difference spectra, and it too seemed to have a full complement of cytochromes. Yet, when succinate was added to crude extracts of the C7SC strain only the cytochrome *b* band was reduced. There was a marked retardation in the appearance of the bands of cytochromes $a + a_3$ and *c*, indicating impaired electron transport between *b* and *c*. Since spectroscopic analysis of strain C7SC revealed cytochrome peaks no different from those of the ancestral strain, Pappenheimer, Howland, and Miller concluded that the missing component in the electron transport chain of the mutant could not be a cytochrome. In 1962, Bishop, Pandya, and King (32) reported that the PW8 strain of *C. diphtheriae* produced 6.6 μmoles of vitamin K_2 per mg (dry weight). This menaquinone was subsequently shown by Scholes and King (275, 277) to have a side chain consisting of eight isoprene units carrying seven double bonds. The established role of a quinone of the vitamin K series (K_9H) in the respiratory chain of *M. phlei* (9) and the fact that actively respiring *C. diphtheriae* produce more menaquinone (MK 8) than do cells slowed down in growth (275) must have suggested to Krogstad and Howland the possibility that one of the causes of the impairment of respiration in the mutant C7SC might be a deficiency in the synthesis of MK. They have found that the $C7_s$ strain makes 23 times more MK than does C7SC. By adding menadione (vitamin K_3,2-methyl-1-4-naphthoquinone) to mixtures used in studying the oxygen uptake of the mutant, they were able to obtain values for oxygen consumption by the mutant which were about the same as those for the wild type (167). In subsequent studies of oxidative phosphorylation by $C7_s$ and C7SC, Kufe and Howland reported that with 4 mM succinate the ΔO (μatoms/mg of protein) for $C7_s$ was 0.65 and for C7SC, 0.35; the P:O values were, respectively, 0.42 and 0.23. With NADH, the ΔO for $C7_s$ was 0.92; for C7SC, 0.87. The ΔO values with tetramethyl-*p*-phenylenediamine (TMPD) plus ascorbate were $C7_s = 0.79$ and C7SC = 0.72 (169). No phosphorylation was detected with TMPD as substrate. Drawing an analogy with electron transport in mitochondria obtained from livers of the rat, in which TMPD passes electrons in that part of the chain to which cytochrome *c* is central (127), the authors conclude that the zero P:O values found with TMPD and extracts of $C7_s$ and C7SC indicate no conservation of energy "in the span from cytochrome *c* to oxygen in *C. diphtheriae*." Their comparative studies with $C7_s$ and C7SC have led them also to conclude that in *C. diphtheriae* there is an "apparent close association between oxidative phosphorylation and menaquinone action." This is in agreement with the studies of Brodie and Adelson (38), which point to a central involvement of menaquinone in the coupling process that joins electron flux and ATP synthesis in *M. phlei*. The authors fail to indicate whether MK, native to $C7_s$, enhances oxygen consumption by preparations from C7SC. In the case of the PW8 strain discussed just following, the endogenous naphthoquinone, MK 8(2H), does not function in a situation in which MK2 restored activity which had previously been destroyed by irradiation.

Scholes and King investigated electron transport in the PW 8 strain of *C. diphtheriae*. Their strain, CN2000 from the Wellcome Research Laboratories, Beckenham, England, has given high yields of toxin over a number of years and has been widely used for the study of toxin production (73, 274, 276). It is a slowly growing strain which contains cytochromes corresponding spectroscopically to types *a*, *b*, and *c* (276, 321). These authors examined the electron-transport mechanism in the particulate and in the supernatant fractions of cells which had been disintegrated by ultrasound at temperatures below 3 C. Succinate oxidase activity was found mainly in the particulate fraction, whereas $NADH_2$ oxidase activity was located mainly in the supernatant fluid, which lacked cytochromes and menaquinone. The activities of the particle and the supernatant fractions in sum were less than that of the starting crude cell extract both with regard to succinate oxidase and $NADH_2$ oxidase. Full activity was restored by recombining particles and supernatant fluid. All three cytochromes were reduced by succinate, lactate, or $NADH_2$, but the substrates did not reduce that portion of cytochrome *b* subject to dithionite reduction. Triton X-100 inhibited oxidation of succinate by the particulate fraction; the addition of succinate resulted in the reduction of cyto-

chrome *b* but cytochromes *a* and *c* were reduced only after some delay. Irradiation at 360 nm completely destroyed menaquinone in the particulate fraction. It also effected a severe decrease in succinate oxidation, whereas succinic dehydrogenase and $NADH_2$ oxidation were little affected. The addition of menaquinones MK-0 and MK-2 restored the succinoxidase activity. MK-8(2H), from the PW8 strain, was without effect. The addition of succinate to the irradiated particulate material caused the immediate partial reduction of cytochrome *b* but only a delayed reduction of cytochromes *a* and *c*. The portion of cytochrome *b* remaining not reduced underwent rapid reduction after the addition of MK-2.

From these results it seems that the corynebacterial respiratory system resides in both the supernatant and the particulate fraction. Certain of its features suggest that it has some things in common with the respiratory chain described for *M. phlei* over the past decade by Brodie and his associates (38). Asano and Brodie suggested three phosphorylating respiratory chains merging into one at the level of cytochrome *b*. (i) The first goes from malate through flavin adenine dinucleotide and involves vitamin K_1 and a phospholipid. (ii) The second transports from NADH through a specific flavoprotein. Both transfer electrons to an endogenous naphthoquinone, MK-9(H), which in turn reduces cytochrome *b*. (iii) The third involves succinate, another specific flavoprotein, and a light-sensitive component required for the reduction of cytochrome *b* (9). Although the routes for oxidative phosphorylation in *M. phlei* are far from fully understood (250), their elucidation thus far points the way for further investigations of the electron transport system in *C. diphtheriae*. A fruitful approach which would seem to be useful in such investigations is that of employing extracts from mutants deficient with respect to two different points in a pathway and determining the exact conditions which allow for functioning of the pathway, i.e., reconstitution by complementation. For example, Azoulay, Puig, and Couchoud-Beaumont (13) used complementation by two extracts from two mutants of *E. coli* K-12 having defects in anaerobic respiration, especially with regard to NADH:nitrate oxidoreductase, and were able to pinpoint the narrow range of conditions of oxygen tension, *p*H, and temperature at which reconstitution takes place.

Deoxyribonuclease. In 1963, Messinova, Vusupova, and Shamsutdinov (197) at the Kazan Medical Institute reported having examined the deoxyribonuclease activity of a collection of toxinogenic and nontoxinogenic strains of *C. diphtheriae*, chosen so as to represent four different serotypes (Russian designations: I, III, IV, and VI) as well as strains of *gravis*, *mitis*, *C. hofmanni*, and *C. xerosis*. It is very interesting indeed that only the toxinogenic strains were found to produce detectable deoxyribonuclease. Enzyme activity was determined by viscosimetric methods and by observing the clearing of DNA in agar gels. Arden recently showed that pairs of toxinogenic and nontoxinogenic *C. diphtheriae* are equally proficient at producing deoxyribonuclease.

Glycoside hydrolases (3.2). All diphtheria bacilli can hydrolyze maltose, but the glucan hydrolase involved has not been characterized. A similar situation exists with regard to the enzyme(s) involved in the hydrolysis of starch. Certain strains of *C. diphtheriae* hydrolyze sucrose (83, 192). This fact is not generally appreciated, and sucrose fermentation is still apparently used for eliminating corynebacteria as candidates for the title *C. diphtheriae* (246). The production of a trehalose-1-glucohydrolase (3.2.1.28) is restricted to those strains designated *C. diphtheriae* var. *ulcerans*. Yet, trehalose is synthesized by all strains of *C. diphtheriae*, and free trehalose accumulates in the medium of cultures of *C. xerosis* (37). No β-galactosidase activity has been found by us for any strains of true corynebacteria.

The *N*-acetylneuraminate glycohydrolase (neuraminidase) (3.2.1.18) of *C. diphtheriae* has been characterized in some detail by Takafumi Moriyama (206), whose findings were confirmed and extended by Marek Jagielski (136). Both of these investigators found much more neuraminidase in cells grown in iron-rich medium than in cells from iron-poor medium. Moriyama has shown that the enzyme is membrane bound and that it is produced by most strains of *C. diphtheriae*. This finding has been extended to strains of *C. ovis* and *C. ulcerans* by Chang (*unpublished data*).

Hemolysin. Certain serological types of *C. diphtheriae* produce a nondiffusing (cell-associated) hemolysin which is active on guinea pig erythrocytes and to a lesser extent on the erythrocytes of rabbits and sheep. The hemolytic activity is inhibited in the presence of cysteine and thioglycolate (114, 313). The presence of the hemolysin appears related to active growth. Penicillin inhibits its production in rapidly growing cells, but less so or not at all in nondividing cells (79).

Nitrate reductase. The capacity to reduce nitrates to nitrites has long been used as a key character in the taxonomy of *C. diphtheriae*

(36). Nitrate reductases are widely distributed among bacteria, and until more is known about the ways in which one nitratase differs from another their use in taxonomy has the same limitations as other trivial and mutable properties. Already, headway is being made in separating certain bacterial nitratases on the basis of their functioning in nitrate assimilation or in nitrate respiration, or both (237–240). Since nitratases occur not uncommonly among corynebacteria, mycobacteria, and nocardias, their role(s) in the physiology of these organisms is probably equal in importance to that played by nitratases in *Aerobacter aerogenes*, *E. coli*, *Pseudomonas aeruginosa*, and perhaps even *Micrococcus denitrificans*. In some of these bacteria, there is now solid evidence that phosphorylation is coupled with nitrate respiration (224, 320). Miyata and Mori (204) recently purified a nitrite reductase from *P. denitrificans*. This copper protein catalyzed nitrite reduction, oxygen consumption in the presence of ascorbate, TMPD, and cytochrome c_{553}, and hydroxylamine oxidation in the presence of nitrite.

The discovery by Hackenthal and associates (103) that chlorate- and perchlorate-resistant mutants of *Bacillus cereus* no longer have the capacity to reduce nitrates led to the development of a useful method for selecting nitratase-negative mutants (*Nred*$^-$ = chlorate resistant = *chl-r*) at the CNRS Laboratory for Bacterial Chemistry in Marseilles (241). Puig and co-workers succeeded in mapping *chl-r* in *E. coli* K-12, (249), and Azoulay, Puig, and Pichinoty examined the alteration in respiratory particles, which is associated with this pleiotropic mutation (13, 14).

The chlorate selection technique has been useful in the isolation of nitratase-negative mutants of *C. diphtheriae* (8); see also the discussion herein of the nitrate-reductase marker in relation to the gene *tox*$^+$.

Porphyrin. In 1931 Coulter and Stone pointed out an apparent relationship between the production of diphtherial toxin and the appearance of porphyrin in the culture medium (55; *see* also reference 44). Since then it has been a common observation that high titers of toxin are almost always accompanied by appreciable amounts of porphyrin. This; porphyrin was sown by Hale, Rawlinson, Gray, Holt, Rimington, and Smith to be coproporphyrin III (104). Coproporphyrin III derives from coproporphyrinogen III, an intermediate in the synthesis of catalases, peroxidases, cytochromes, the heme of haemoglobin, and the Mg-protoporphyrin of bacterial chlorophyll (174). The incorporation of N^{15}-glycine into the intracellular hemes and into the porphyrins excreted by *C. diphtheriae* in these experiments of Hale and associates offered the first evidence that in bacteria the pathway for heme biosynthesis was similar to that occurring in "higher forms". Both toxinogenic and nontoxinogenic diphtheria bacilli under identical cultural conditions accumulate in the medium similar amounts of coproporphyrin III (323). Porphyrin[4] "excretion" appears to be common among certain genera of bacteria (174, 272, 296). An observation of Mary Wheeler, that whereas cultures of toxinogenic and nontoxinogenic strains of *C. diphtheriae* as well as strains of *C. ovis*, *C. ulcerans*, and *C. hoagii* accumulated porphyrins, *C. xerosis* and *C. hoffmanii* did not, would seem to bear further examination (310).

DNA-Containing Corynebacteriophages

Corynebacteriophages and the gene *tox*. Freeman, in 1951, made the remarkable discovery that nontoxinogenic *C. diphtheriae* became toxinogenic after infection with a bacteriophage (81) which later came to be named β (17, 24). Groman (94) and others (193) readily confirmed this discovery. Several examples of modification of bacterial genomes (so-called lysogenic conversion) following lysogenization by certain bacteriophages are now on record (*see* Fig. 1, 15, 16). Unfortunately, many claims to having found this kind of modification are without real proof, and reviewers who publicize such claims in the absence of solid evidence are compounding one of the crimes against our forests. Since pseudolysogenic associations (Lwoff, 1953: clones of bacteria contaminated with bacteriophages)

[4] Sickles and O'Leary (281) in 1968 had the following to say about porphyrin and *C. diphtheriae*: "It is clear that when β-prophage-infected cells of *C. diphtheriae* are grown in an environment with a suitably limited supply of iron, both toxin protein and porphyrin are secreted by the cells. This striking concatenation of iron, porphyrin and proteinaceous toxin has led to the concept that diphtherial toxin may be the protein component of *C. diphtheriae* cytochrome." The idea that toxin might be the protein moiety of diphtherial cytochrome *b* (225, 231) has influenced the research on toxin production by *C. diphtheriae* since 1947 more than any other one concept. It all began when Pappenheimer found Fe:porphyrin:toxin ratios of 4:4:1 in supernatant fluids from still-grown cultures of the PW8 strain. Hata, in 1951, working with a number of diphtheria bacilli isolated in the Tokyo area, pointed out that the 4:4:1 ratio found by Pappenheimer in the case of the PW8 strain could not be generalized to other strains of *C. diphtheriae* (106, 109). Clarke and Clarke found ratios of 2:2:1 (47–49) for another strain of *C. diphtheriae* used by them. Their data also suggest that the heme of cytochrome *b* in iron: sufficient *C. diphtheriae* accounts for only 10% of the total porphyrin such bacilli would have excreted had they been in a state of iron deficiency. What will always be of value in the iron-porphyrin:toxin story is the amount of good research it stimulated. After all, "what is wanted is not the will to believe but the wish to find out, which is its exact opposite." (From Lord Bertrand Russell, *Free Thought and Official Propaganda*.)

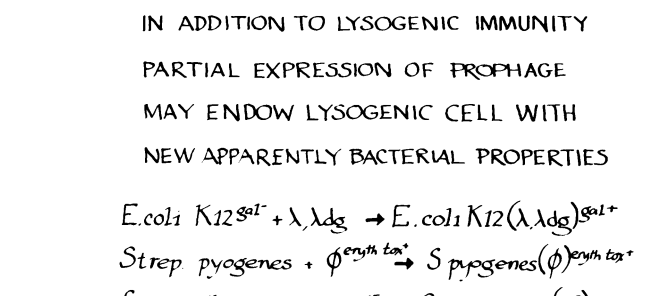

FIG. 15. *Of these examples, only in the case of λdg (carrying the genes governing the synthesis of galactokinase, galactose-1-P uridyl transferase and UDP-galactose epimerase) has it been established that the genes controlling prophage-effected bacterial syntheses are of bacterial origin. Each of these three phage-controlled changes in the bacterial genome is an example of "lysogenic conversion." Elimination of the prophage in each case results in a loss of the phage-related property (see Fig. 1 and 16). For further details, see reference 111.*

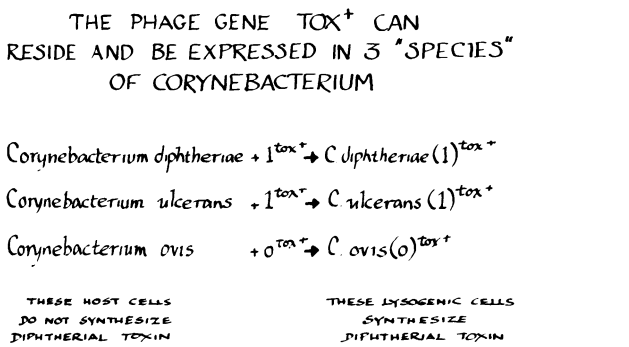

FIG. 16. *Expression of the gene tox in corynebacterial "species." Traditionally, C. diphtheriae and C. ulcerans have been associated with infections of man, whereas C. ovis has been associated with infections of sheep (for exceptions see text). When any one of these corynebacteria is infected with certain tox+-carrying bacteriophages, it produces diphtherial toxin (see also Fig. 1 and 15).*

mimic true lysogeny, any claim to establishing lysogeny must be backed up by at least the evidence that (i) the phage stocks employed were bacteria-free and (ii) the passage of the "lysogenic clones" in the presence of antiphage serum did not affect their "lysogenic" condition.[5]

[5] In 1952, Hewitt hinted that staphylococcal phages converted nontoxinogenic *C. diphtheriae* to toxinogeny (*see* 115 and page 321 of reference 187). In 1953, through the interest and cooperation of André Lwoff, Hewitt's phages and bacteria were used at the Pasteur Institute for repeating Hewitt's experiments. Our findings were simply this: when bacteria-free stocks of staphylococcal phages and corynebacterial phages were employed, plaques were formed on staphylococci only by staphylococcal phages and on corynebacteria only by corynebacteriophages. However, when drops of high-titer stocks of either of the phages were placed on lawns of either of the indicator bacteria, clearing sometimes occurred. This is not an uncommon effect. Most stocks of phage contain murolytic enzymes and such enzymes lyse any bacteria having interpeptide bridges susceptible to their hydrolytic action. One more point important to understanding Hewitt's results: certain of his strains of staphylococci were capable of inhibiting the growth of *C. diphtheriae*. When such strains were grown in liquid culture with toxinogenic diphtheria bacilli, the diphtheria bacilli were so inhibited that they could not be found in Gram-stained smears and only with difficulty when the mixed broth cultures were streaked on chocolate plates. However, such apparently staphylococcal cultures contained enough diphtherial toxin to produce positive skin tests in rabbits or in guinea pigs.

Recently Bakulina (19), with little data and no reference to Hewitt, made essentially the same claim as Hewitt for the action of staphylococcal and streptococcal phages on *C. diphtheriae*. Jones and Sneath considered this report possible "gene evidence" for the existence of closer relationship between *Staphylococcus, Streptococcus, Corynebacterium, Propionibacterium,* and the "corynebacteria" pathogenic for plants. Stratienko (285), working with staphylococcal phages, streptococcal phages, corynebacteriophages and their host bacteria including nontoxinogenic strains of *C. diphtheriae* and *C. hofmanni*, obtained findings just the opposite of those of Bakulina.

A mutant of phage β, a typical corynebacteriophage, is shown in Fig. 17 in which also are illustrated the plaques it forms, with their characteristic halos. In Fig. 6 are shown cells of the indicator strain $C7_s(-)^{tox-}$ ravaged by virulent corynebacteriophage and the enzymes associated with phage liberation. (For details regarding the growth of corynebacteriophages see references 121, 122.)

Matsuda showed that β-phage was a DNA phage (191). Holmes recently worked out a mating system for β and related corynebacteriophages (121). He also studied *tox*$^+$ phages not closely related to β.

Using the markers *h* (host range), *imm* (lysogenic immunity), *tox*, *c* (clear plaque), and *h'* (extended host range), Holmes showed that in crosses between phages β^{tox+} and γ^{tox-}, *tox* behaves as though it were close to *h* and a map order of *-h-tox-imm*$^{\beta}$*-c-h'-* seems well established for phage β^{tox+}. Thus, *tox* is a corynebacteriophage gene.

More recently, Holmes examined morphologically and serologically distinct phages carrying *tox* for their capacity to undergo genetic recombination as a measure of their relatedness. It was expected that *tox* phages would probably all undergo genetic recombination, but this was not the case. In fact, *tox* is found in bacteriophages which are morphologically distinct, in phages which are serologically distinct, and among phages which cannot recombine genetically. When hybrid phages differing only in the presence or absence of *tox* are compared as to efficiency of absorption, latent period, burst size, stability in storage, *etc.*, they seem to be

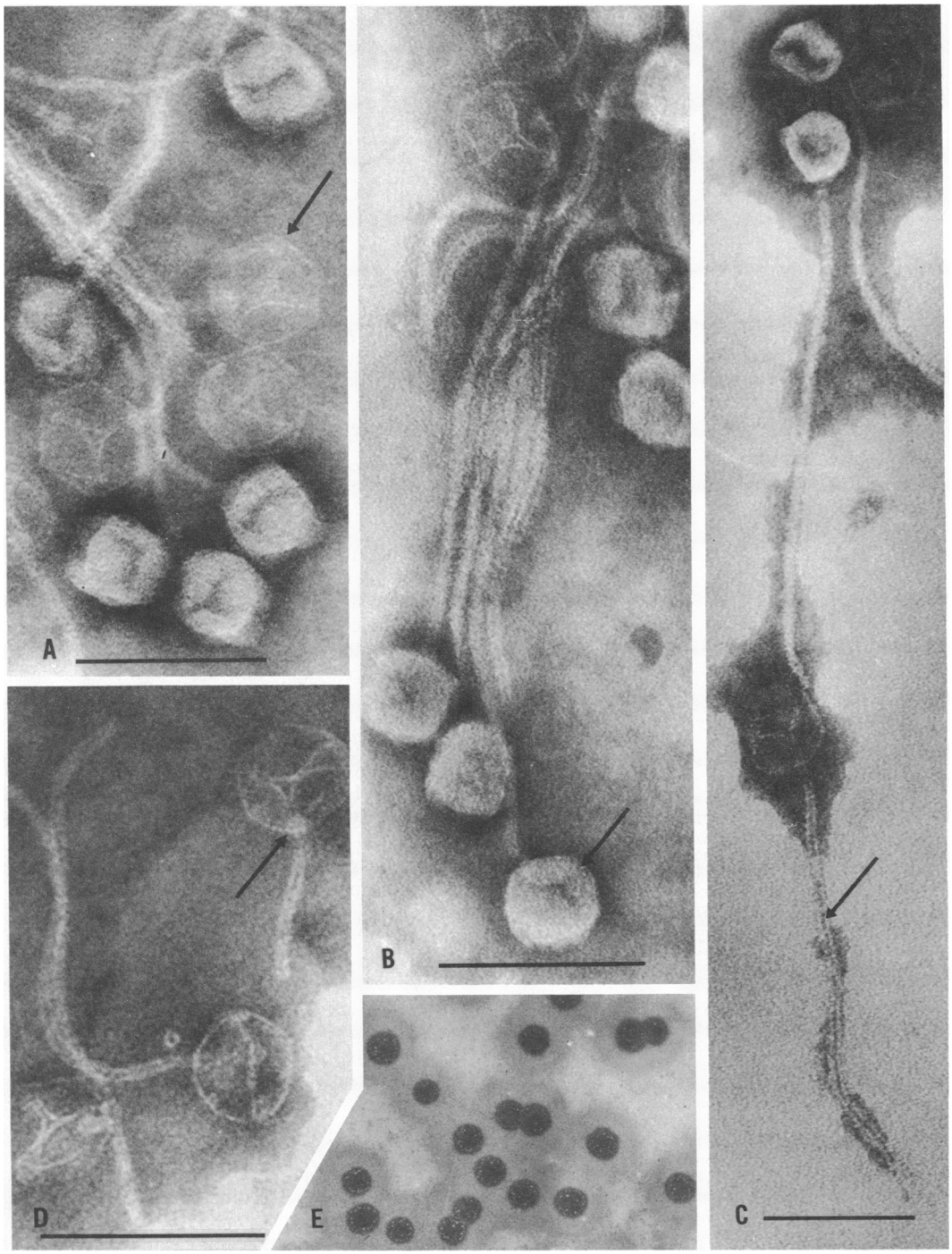

FIG. 17. *(A–D) Electron micrographs of bacteriophage* $\beta^{hv64tox^+}$ *(A) filled and empty heads; (B) full heads with long unadorned tails; (C) tail groove; (D) point where head joins tail; (E) plaques formed by* $\beta^{hv64tox^+}$ *on indicator strain* $C7_s(-)^{tox^-}$ *(× 2.5) showing ring of resistant colonies just within central clearing and surrounding halos. Initial magnifications for A–D, 39,000 to 80,000. Marker = ±100 nm. Suspensions in neutral potassium phosphotungstate were mounted on carbon-supported collodion films and examined under a Siemens Elmiskop IA electron microscope. For these pictures, we are much indebted to John Freer, Aina Neimanis, and Charles Harman. (Reprinted from the Journal of Bacteriology.)*

alike (122). In other words, *tox* seems to endow phages with no special advantage(s).

Stability of integration of the *tox* prophages. Although it has been reported that toxinogenic strains of *C. diphtheriae* may be rendered nontoxinogenic by loss of their tox^+-containing prophages (7), our experience has been that such prophages are very stably integrated and that only in pseudolysogenic strains, carrier cultures, does one observe loss of toxinogenicity. In fact, no prophage loss has been observed in strains of $C4_s(\beta)^{tox+}$ and $C7_s(\beta)^{tox+}$ which have been under cultivation in our laboratory for over 18 years. In this connection, the PW8 strain is especially interesting. It is a rough bacterium lacking receptors for any of the known corynebacteriophages. Therefore, should it lose its *tox*-carrying prophage, reinfection is unlikely. This bacterium has been in continuous cultivation for 74 years. Recently, we have examined five strains of PW8 maintained in laboratories in various parts of the world and found them all to be toxinogenic and lysogenic (172). Thus, the corynebacteriophages examined seem stably integrated with the host genome, offering some of the best examples of this kind of stability on record.

Maximescu et al. (195), using different corynebacteriophages, showed that the gene *tox* can reside in and be expressed in strains of *C. diphtheriae* var. *ulcerans* and *C. ovis* (195), and this finding was confirmed by Goldzimer and Arden (8, 86; see Fig. 16).

Product of the gene *tox*. The simple protein diphtherial toxin is either directly or indirectly the product of the *tox* gene. It has a molecular weight of about 64,000 (150, 154, 229, 244, 256, 259). Its amino acid content is not remarkable (253), and it is readily crystallized from complex media (154, 244, 245) but not from defined media (Hirai and Barksdale, *unpublished data*). Photographs of crystalline toxin are shown in Fig. 18. Standard toxin has a sedimentation coefficient of 4.2*S*. No function for toxin has yet been found in either the lysogenic cell or the lysing cell.

Although there is as yet no understanding of what toxin is, there is considerable information as to what it is not. For example, it has been proposed that *tox* was linked to the nitratase marker of *C. diphtheriae* and that when phage β^{tox+} was integrated into the genomes of such $Nred^-$ corynebacteria as *C. ulcerans* and *C. belfanti* (101), those bacteria became nitratase positive and tox^+. These experiments seem not reproducible with the strains originally employed, and Arden and Goldzimer (8, 86) showed that when the phages β^{tox+}, 1^{tox+} or β^{hvtox+} are introduced into $Nred^-$ strains such as *C. diphtheriae* var. *ulcerans* 603, and $C7_s^{Nred-}$, the resultant lysogenic strains or lysates, or both, are toxin producers and are $Nred^-$. The interesting observation of Warren and Spearing (305) of an association between diphtherial toxin and neuraminidase activity and antitoxin and antineuraminidase activity posed the question of whether toxin might originate from the formation of dimers or trimers of neuraminidase lacking the capacity to turnover their substrates. Moriyama (206) made a careful study of this possibility and found no link between neuraminidase and diphtherial toxin.

Diphtherial toxin is lethal for man and animals in doses of 130 ng per kg of body weight (23, 312). The elucidation of what is currently considered to be the way in which toxin exerts its lethal effect began with the publication by Lennox and Kaplan (179) of a list of cultured animal cells, some of which were sensitive to toxin. Strauss and Hendee, using HeLa cells (strain S3), established (i) that in intoxicated HeLa cells glycolysis and aerobic respiration continued at a normal rate for many hours, whereas (ii) protein synthesis, as reflected by the inability to incorporate radioactive methionine, was stopped very early. (iii) The intoxicated cells developed visible blebs within 4 hr and underwent destruction about 7 hr after exposure to toxin. Because low temperatures blocked intoxication of HeLa cells, a means was available to these investigators to examine the initial steps in the interaction of toxin and cells. Adsorption of toxin by the cell was very rapid (287). Subsequently, Strauss showed that toxin was without effect on oxidative phosphorylation in HeLa cells (286).

In 1960, Kato and Pappenheimer (151) and, in 1962, Kato (149) offered additional evidence for an effect of toxin on mammalian protein synthesis. Collier and Pappenheimer (52) found that NAD was required for the inhibition of protein synthesis in cell-free systems (from HeLa cells and rabbit reticulocytes). Later, Collier (50, 53) and Goor and Pappenheimer (89) showed that toxin specifically inactivated aminoacyl transferase II, a soluble translocase involved in the messenger-RNA-directed growth of polypeptide chains. Goor, Pappenheimer, and Ames (90) next showed that, in suitable concentrations, nicotinamide could reverse the action of toxin (*see* 85). In 1968, Honjo, Nishizuka, Hayaishi, and Kato (123) presented evidence for the mechanism of the inactivation of transferase II by toxin. According to their findings toxin catalyzes the transfer of the ADP-ribose portion of NAD to transferase II, thereby bringing about its inactivation. These authors

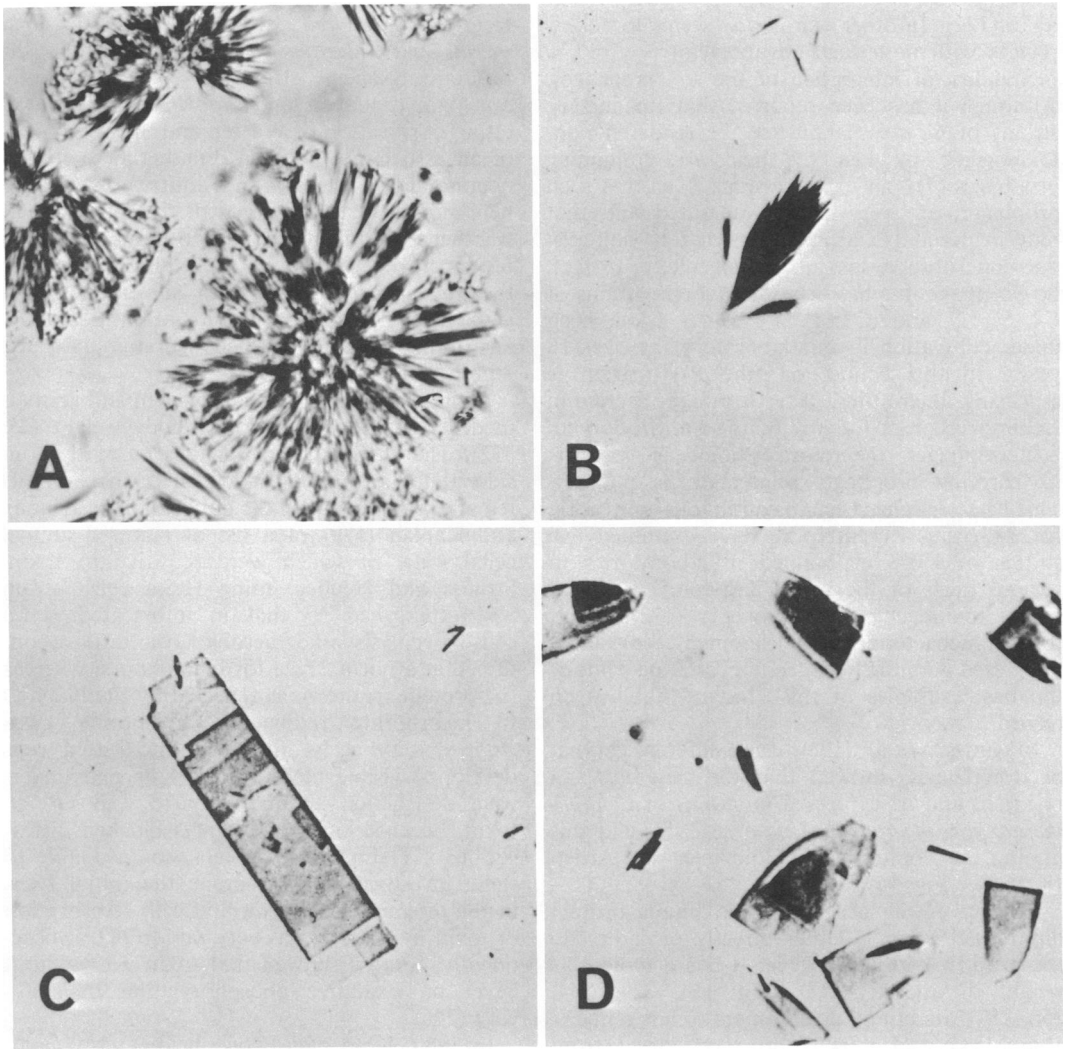

FIG. 18. *Crystals of diphtherial toxin (from Pope and Stevens, reference 245). A, Rosettes from $(NH_4)_2SO_4$; B, needles once crystallized from K_2HPO_4; C, plates recrystallized from K_2HPO_4; D, Crystalline shields from $(NH_4)_2SO_4$; Magnification about 1,200. (Reprinted with the permission of the editors of the British Journal of Experimental Pathology.)*

showed that a stoichiometric amount of nicotinamide was released and that the reaction was reversible. Further, the authors gave evidence which shows that the ribosome-dependent guanosine triphosphatase activity associated with transferase II is inactivated in the same toxin-induced ADP-ribosylation reaction. There is one very unphysiological aspect of this beautiful work: (i) the ADP-ribosylation reaction is optimal at *p*H 8.2; (ii) the reverse reaction goes at *p*H 5.3 and hardly at all at *p*H 7.0 (124).

Collier and Goor have independently offered evidence for more than one molecular species of diphtherial toxin. Goor (88) described a heavy molecule having a sedimentation coefficient of 6.8*S*. Recently, Relyveld has produced evidence which indicates that Goor's heavy toxin may be a product of ammonium sulfate fractionation (257). Collier has described a light toxin molecule with a sedimentation coefficient of 2.5*S*. Collier's findings are in keeping with the observation of Bizzini, Prudhomme, Turpin, and Raynaud (33) that the sedimentation coefficient of toxin is reduced from 4.2 to 2.0*S* after treatment with disulfite and with the finding of Iskierko (135) that toxin appears to have more than one equivalent of amino-terminal acid per

64,000 molecular weight. Collier (51–53) offered evidence which suggests that it is the subunits and not toxin which bring protein synthesizing systems to a halt. The subunits, 2.5*S* as opposed to 4.2*S* for toxin, are (i) seven times as active as toxin in ADP-to-transferase II tie-up activity and (ii) are nontoxic for animals and animal cells. [Collier (*personal communication*) says that the 2.5*S* subunit, as one might expect, is capable of acting as a blocking agent in the specific reaction between toxin and antitoxin.]

Thus, at this time one can only speculate as to the role of the subunit in the extreme toxicity of diphtherial toxin. There is as yet no evidence for a role of toxin in the synthesis of corynebacteriophages, and although *tox* is a phage gene there is no indication of what its relation is to the biogenesis of toxin. The molecular configurations for which toxin shows specificity would seem just now to be the only clue to its possible mode of action in animal cells and perhaps to its origins from the lysogenic bacterial cell. In this connection, the finding of Agner (4) that aniline, *o*-toluidine, pyrocatechol, resacetophenone, benzidine, tyramine, indole, and iodide (but not methylaniline, *p*-toluidine, resorcinol, phloroacetophenone, methyl salicylate, *o*-tolidine, tyrosine, skatole, or tryptophan) could act as cofactors in the peroxidatic detoxification of toxin (3) may have some bearing on the nature of the toxic site(s) of the diphtherial toxin molecule. Similarly, the finding of Kim and Groman (158) that ammonium ion and certain amines inhibit the toxicity of diphtherial toxin for HeLa cells may offer a clue to the chemical nature of the site to which diphtherial toxin fixes on the mammalian cell.

Expression of *Tox*

The synthesis of diphtherial toxin is per se the expression of the gene *tox*. This can occur either in a nontoxinogenic cell being lysed by phage or in a lysogenic, toxinogenic cell.

Synthesis of toxin in one cycle of viral growth. Morihiro Matsuda (190, 191) devised an experimental system for infecting nontoxinogenic *C. diphtheriae* with a virulent phage carrying the gene *tox* and used it to observe the expression of *tox* during a single cycle of viral growth. In Fig. 19 are shown the results of an experiment in which the synthesis of viral DNA is accompanied by the intracellular appearance of toxin at between 7 and 14 min postinfection. Extracellular toxin is released before the first phage particles are liberated. Toxin ceases to be synthesized at the time the cells begin to succumb to lysis (*see* optical density curves in Fig. 20). In the experiment shown in Fig. 19, the number of infected cells was 4×10^9/ml. The total yield of toxin was 8 flocculation units (Lf) or $8 \times 15 \times 10^{12}$ molecules/ml. Thus the yield of toxin per cell was $120 \times 10^{12}/4 \times 10^9 = 30 \times 10^3$ molecules. The total time to reach maximal yields in these experiments was 3 hr. About 10,000 molecules were made per cell per hr. This value compares favorably with the yields shown in Fig. 20 in which the number of molecules per cell per hr is about 5,800. Matsuda showed that the toxin made in one cycle of viral growth was immunochemically indistinguishable from the type toxin produced by the PW8 strain (190). The evidence for this fact comprises Fig. 21. Agents or conditions which prevent viral maturation, or which in some other way delay lysis of the toxinogenic cell, extend the duration of toxin synthesis and thereby enhance yields of toxin. This enhancing delay has been brought about experimentally through the use of proflavine (23, 191) and, somewhat unwittingly, under conditions of limited iron (*see* Fig. 20). It has been pointed out

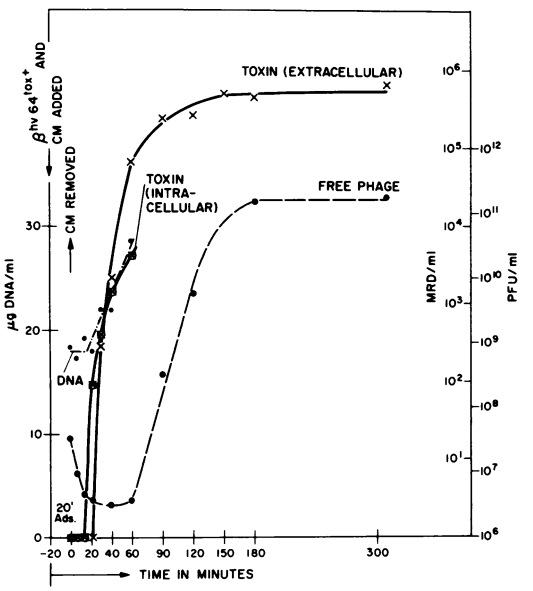

FIG. 19. *Synthesis of DNA, bacteriophage, and diphtherial toxin in nontoxinogenic C. diphtheriae, C7$_s$(−)$^{tox^-}$, after infection with purified (toxin-free) phage particles of phage β$^{hv64tox^+}$ (see also Fig. 2, 6, and 17). Adsorption of phages to bacteria took place in the presence of chloramphenicol (CM; 125 μg/ml). After the adsorption period, the infected cells were washed free of chloramphenicol and unadsorbed phage, resuspended in fresh medium, and incubated at 36 C in a shaking water bath. Samples were taken as indicated and assayed for intracellular toxin (⊠), extracellular toxin (×), DNA (●) and bacteriophage (PFU; ●). 1 MRD = 0.000018 μg of toxin protein. Reprinted from the Journal of Bacteriology.*

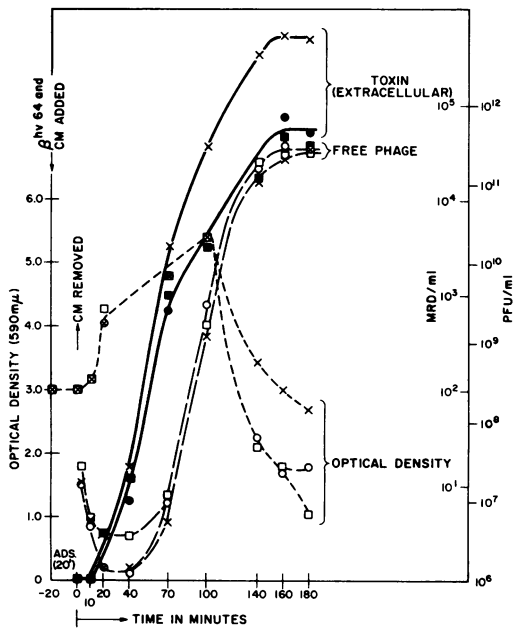

FIG. 20. *Effect of iron on the synthesis of diphtherial toxin by C. diphtheriae, strain $C7_s(-)^{tox^-}$, infected with hypervirulent corynebacteriophage, $\beta^{hv64tox^+}$. Portions of a culture of $C7_s(-)^{tox^-}$ (grown to an optical density of 4.7 in deferrated medium supplemented with 0.1 μg of iron/ml) were diluted in (i) deferrated medium to which no iron was added and (ii) deferrated medium supplemented with 3.0 μg of iron/ml, in both instances to give an optical density of 0.3, and 30-ml samples of each were incubated in 300-ml flasks. These low-iron and high-iron cells were allowed to grow to optical density 2.85 (3.7×10^9 cells/ml); then each was combined with 17.5 ml of a suspension of β^{hv64} (4.3×10^{10} PFU/ml). Adsorption was allowed to proceed for 20 min in the presence of 5×10^{-3} M calcium chloride and 125 μg of chloramphenicol/ml. Infected cells were then washed in chilled deferrated PGT medium containing 4% maltose and were resuspended as follows: (×) infected low-iron cells were resuspended in deferrated medium; (○) a portion of infected high-iron cells was resuspended in deferrated medium; (□) a second portion of infected high-iron cells was resuspended in medium containing 3.0 μg of iron/ml. Samples were taken for optical density, PFU, and extracellular toxin at the indicated times. Effective multiplicity of infection was 2.1 (from Matsuda and Barksdale, reference 191). (Reprinted with the permission of the editors of Nature.)*

in the section on iron phenotypes that cells grown in low iron exhibit a lengthening of their latent period of viral multiplication. In other words, they lyse more slowly than cells supplied with an adequate amount of iron. Matsuda has shown that the yields of toxin are markedly enhanced in the low-iron cells showing delayed lysis (*see* Fig. 20).

Synthesis of toxin by the lysogenic, toxinogenic *C. diphtheriae* strain PW8. The Park Williams 8 strain, $PW8_r(P)^{tox^+}$, carries a prophage, P, which at one time was thought by this reviewer to be a defective phage because of its infinitely low plaque-forming ability on the indicator strain $C7_s(-)^{tox^-}$. Maximescu, however, found that P phage would form a thousand times as many plaques on a strain of *C. diphtheriae* var. *ulcerans* (194). Lampidis has shown that P phage is restricted in $C7_s$ but not in a strain of *ulcerans* called *603* (172). In the PW8 strain, then, the gene *tox*$^+$ resides in prophage P. Since this strain makes amounts of toxin (detectable by in vitro methods) only under conditions of iron limitation, those investigators who work with it have often been puzzled by the relationship(s) existing among growth, iron deficiency, and toxin production. Edwards has addressed himself to the problem of how growth of PW8 strain CN2000 as measured by viable count and bacterial nitrogen relates to the accumulation of protein (toxin) in the culture medium. He reviews the opinions of Mitsuhashi et al. (203) that toxin production parallels growth, of Raynaud et al. (254) that bacterial nitrogen and toxin increase together, of Nishida (220) who called attention to the possible accumulation of dead cells among the total numbers comprising the growth and making the toxin, and of Pappenheimer (226) who pointed out that at the time of toxin production the cells had exhausted their iron supply. Edwards himself seems to wish to conclude that the continued secretion of extracellular protein depends upon the viability of the cells. Probably integrity is a better term than viability. Viability should be equivalent to "colony-forming ability." Elsewhere in his paper, Edwards states that "part of the discrepancy in the present work appears to arise from changes in the size of the organisms at different times during growth. For example in one culture (SC 1499) the organisms which were initially about 3.7 μ long were 2.0 μ after growth for 12 hr and then elongated steadily until they were 5 μ long at 48 hr." Is he here writing about the low-iron phenotype (*see* section on iron phenotypes)? Are those elongating cells dying cells? Is toxin synthesis in these populations to be attributed to an ever increasing minority of cells which are producing toxin among a majority which is not producing toxin? Edwards has published three very clear and thoughtful papers relating to growth and toxin production by this diphtheria bacillus. The data presented in Fig. 22 and 23 are from his work. He has looked at toxin production in relation to viable count [as related to bacterial nitrogen and phosphorus (Fig. 22) and as related to levels of catalase and porphyrin (Fig. 23; *see* section on enzymes and pigments

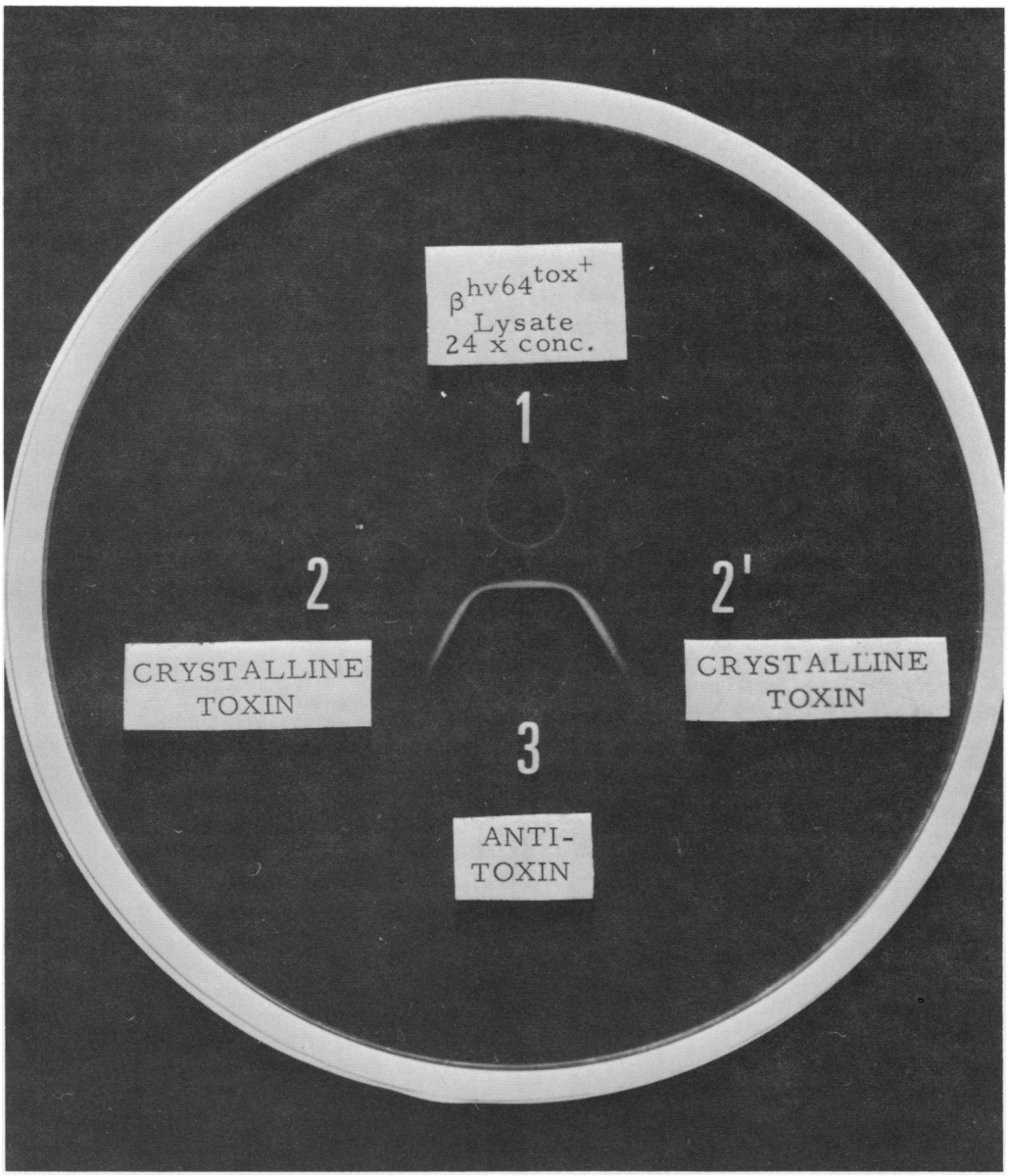

FIG. 21. *Fused antigen-antibody bands resulting from diffusion in agar of antitoxin (see below), crystalline diphtherial toxin (Pope), and toxin produced under the direction of corynebacteriophage, $\beta^{hv64tox^+}$, during one cycle of growth in nontoxinogenic C. diphtheriae, strain $C7_s(-)^{tox^-}$. The wells labeled, 1, 2, 2', and 3 contained the following reactants in volumes of 0.2 ml: (1) $\beta^{hv64tox^+}.C7_s(-)^{tox^-}$ lysate (24 × concentrated as 0 to 80% saturated ammonium sulfate fraction); (2 and 2') 20 Lf of 10 × crystallized diphtherial toxin, (Pope); (3) 20 au of diphtherial antitoxin (rabbit) produced with toxoid derived from crystalline toxin. Contact print was made after incubation for 3 days at 37 C. Prolonged incubation resulted in some thickening of the bands; no spurs were formed (from Matsuda and Barksdale, reference 161). (Reprinted with the permission of the editors of Nature.)*

for discussion of porphyrin)]. Edwards used flocculation as a means of detecting toxin, and so he required the presence of 500,000 times more toxin than did Matsuda in order to detect its early appearance in his cultures. [Matsuda's unit of measurement was the MRD (minimum reacting dose in the skin of the rabbit); 1 MRD = 10^{-5} flocculating units or Lf. 1 Lf = 1.75 μg of toxin protein. The least amount of toxin detectable by flocculating a small sample is about 5 Lf.] Nevertheless, it is clear from an inspection of Fig. 23 that at 18 hr, when the viable count

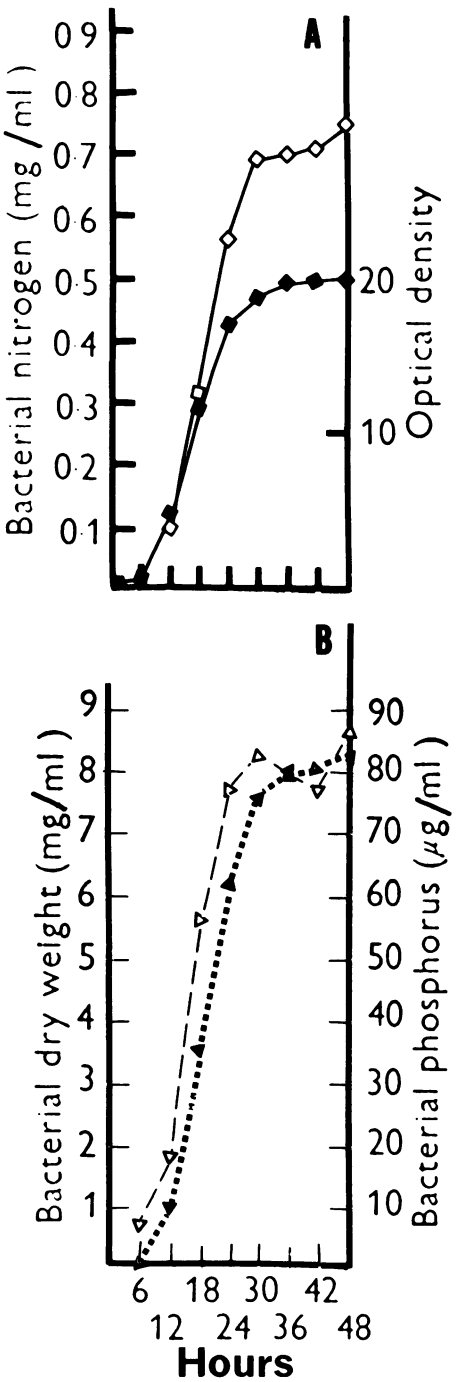

FIG. 22. (*A*) *Arithmetic plots of the growth of C. diphtheriae in submerged culture as measured by optical density* (■) *and bacterial nitrogen* (□). (*B*) *A separate experiment in which growth was followed by measuring the dry weight* (▲) *and the bacterial phosphorous* △. (*After D. C. Edwards, 1960.*)

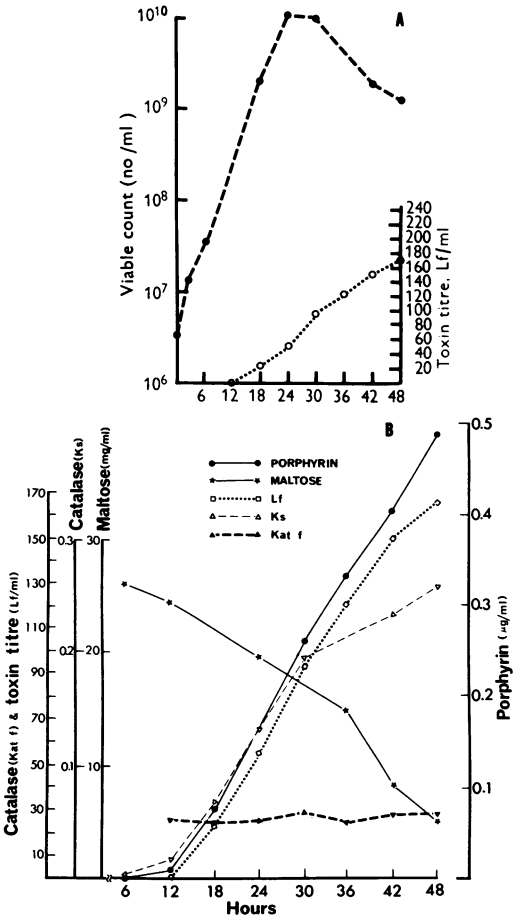

FIG. 23. (*A*) *Events associated with the expression of the gene tox in C. diphtheriae* (*PW8, strain CN 2000*) *growing in submerged culture in a complex medium. The relation of viable count to titers of toxin.* (*B*) *Events associated with the expression of the gene tox in C. diphtheriae* (*PW8, strain CN 2000*) *growing in submerged culture in a complex medium. The consumption of maltose and the production of catalase, porphyrin* (*as coproporphyrin III*), *and toxin.* [*After D. C. Edwards, 1960* (*73*).]

had reached about 3 × 10^9, already the toxin level was 20 Lf (or 300 × 10^{12} molecules/ml or 10,000 molecules per cell). Since it took 18 hr to accumulate this amount of toxin, each cell would have had to make only 550 molecules per cell per hr, provided all of the cells, from the outset, were synthesizing toxin.

Edwards noted that the viable count does not always agree with optical density, and his discussion and his data raise at least the following questions: (i) is there a fraction of the population which makes toxin or does all of the population make toxin, and (ii) are cells which are incapable of giving rise to a colony (nonviable = dying)

capable of synthesizing toxin? With regard to the first question, it is obvious from Matsuda's data that a cell can easily make 5,000 molecules of toxin per hr. Therefore 5×10^8 cells in the population studied by Edwards could, in 18 hr, synthesize 18 Lf of toxin. Such a small number of cells would be completely masked by the majority of the population. With regard to viability and synthesis of toxin, in 1961, Barksdale et al. (24) raised the question of whether toxin could be synthesized in the absence of bacterial DNA synthesis and said that experiments designed to answer this question "were in progress." They were then in progress with Masahiko Yoneda in Osaka and they were completed in New York with Morihiro Matsuda. They involved (i) inactivating bacterial DNA with mitomycin C (MC), and (ii) observing the synthesis of toxin, DNA, RNA, and protein in both the MC-treated and the control cells. The pattern of the data obtained comprises Fig. 24, in which it is perfectly clear that in cells committed to the synthesis of toxin, toxin synthesis as well as RNA synthesis continued in the absence of bacterial DNA synthesis. The slight rise in DNA after treatment with mitomycin C (*see* Fig. 24) presumably is viral DNA. The results of these experiments should have been expected because it was already known that ultraviolet light-induced cells go on making toxin and that the phage-infected cell makes toxin up to the time of lysis. Each of these is in effect a non-colony-forming cell.

"Il n'y'a réellement dans la Nature que les individus."
Lamarck, *Discours*

The matter of getting an answer to the question of which cells in populations of the PW8 strain make toxin is being undertaken by Kwang Shin Kim in our laboratory. In experiments in which he gets 10 Lf/optical density unit, most of the cells look like those shown in Fig. 8A. A quick comparison of these cells with those in Fig. 2, 6, 7, and 10 indicates their unusual nature. Ways are now being worked out to separate such unusual cells from the more normal looking cells in the toxin-synthesizing population and to examine them en masse and individually for active secretion of toxin.

In any population of toxinogenic *C. diphtheriae* growing under optimal conditions (no iron deprivation), a small fraction of the population is always making toxin. These toxin producers are undoubtedly cells which are under-going spontaneous induction of prophage to phage. It has long been known that only about 15% of such cells yield plaque-forming particles (27).

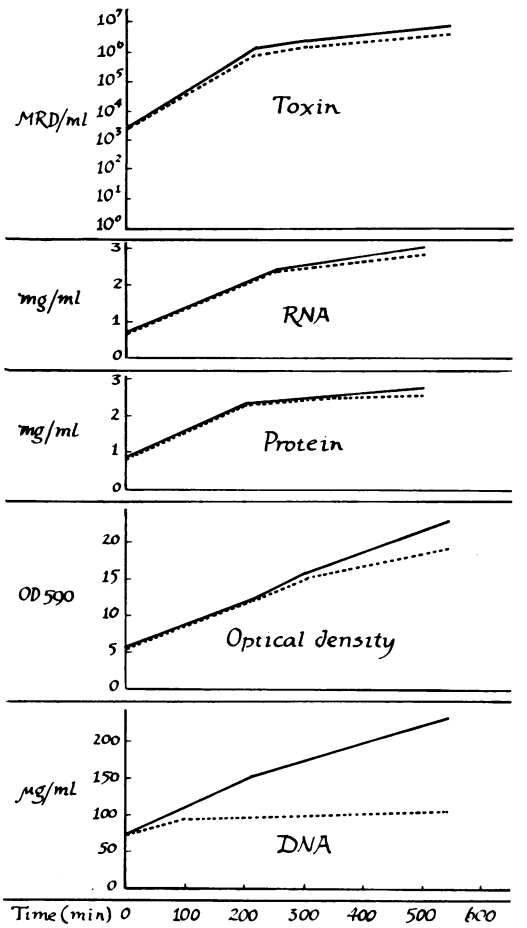

FIG. 24. *Increase in optical density and the synthesis of toxin by cultures of the PW8$_r$(P)$^{tox^+}$ strain of C. diphtheriae treated (dotted line) with and not treated (solid line) with mitomycin C. (Exposure to mitomycin-C, 0.1 μg/optical density 0.3, was for 1 hr. Cells were then washed and resuspended in fresh medium, and sampled from time to time to determine optical density, DNA, RNA, protein, and toxin protein. Toxin was measured as MRD, when the levels were small, and as flocculating units (Lf) when the levels were above 8 μg of toxin protein/ml; 10^5MRD = 1 Lf = 1.75 μg of toxin protein.) This composite plot is from unpublished data of Matsuda and Barksdale. For data on the synthesis of toxin by nondividing cells, see Hirai, Uchida, Shinmen, and Yoneda (119).*

APPENDIX

In the introduction, ample evidence was given to justify our considering the members of the *CMN* group as a family. Now we come to the matter of which of the organisms in the assemblage called the genus *Corynebacterium* really belong there. *C. diphtheriae* does belong because it is the type species. A new description

of this organism, based more on what it can do and less on how it appears, follows.

Proposed Changes in the Official Description of *Corynebacterium diphtheriae* (Flügge, 1886) Lehman and Neumann, 1896.

Facultatively aerobic, gram-positive to gram-variable, nonsporulating, nonmotile, rodlike, tapered bacteria. Actively growing cells appear as doublets tapered from their septal ends. Club-shaped phenotypes occur in old cultures and on inadequate media. Intracellular polyphosphate granules, formed on serum slants rich in phosphate, can be revealed by staining with the metachromatic dyes, Toluidine Blue and methylene blue. Most strains ferment glucose, maltose, and dextrin; fewer ferment starch, still fewer sucrose. GC content about 55%. Cell walls are distinguished by having meso-α,ϵ-DAP in conjunction with arabinogalactan, corynemycolic, and corynemycolenic acids and trehalose (dimycolate). O (polysaccharide) antigen-cross reacts with O antigens of *Mycobacterium* and *Nocardia*. Specific K antigens (protein) are basis for serotyping. Corynebacteriophages may be used for further typing members of the genus and for distinguishing corynebacteria from mycobacteria and nocardias. Certain lysogenic strains harboring prophages carrying the *tox* gene produce the immunologically distinct protein, diphtherial toxin, molecular weight 64,000, 4.2*S*. Subunits of toxin, 2.5*S*, obtained by treatment with dithiothreitol, bring about the ribosylation of the mammalian translocase, transferase II. Most strains produce a neuraminidase (sialidase) which cleaves neuraminlactose to lactose and N-acetyl-neuraminic acid. Neotype: *Corynebacterium diphtheriae*, strain(s) $C7_s(-)^{tox^-}$ and $C7_s(\beta)^{tox^+}$.

Presumably, the above description leaves us with at least the following members of the genus: "*C. belfanti*," *C. bovis*, *C. equi*, *C. hoagii*, *C. kutscheri*, *C. minutissimum*, *C. murisepticum*, *C. pseudodiphthericum* (*hofmanni*), *C. pseudotuberculosis* (*ovis*), *C. renale*, and *C. xerosis*. To straighten out the interrelationships of these members of the genus, we need additional information such as that which has made possible the above revised description of *C. diphtheriae*. Such information is not now available.

"*C. belfanti*" has a characteristic corynebacterial wall (Arden, *unpublished data*), adsorbs standard corynebacterial phages but is not lysed by them, comprises at least two K antigen groups which to a minor extent cross-react (100) with Huang's (129) serotype K(D5), and are by definition nitratase negative (*see* also discussion of the gene *tox*). All strains tested possess neuraminidase activity.

C. diphtheriae var. *ulcerans* is a starch and a trehalose-fermenting, gelatin-liquefying bacterium which is remarkably virulent (invasive) for the rabbit. Jebb (138) described a starch-negative mutant from a human case of "pharyngitis." When infected with a tox^+ bacteriophage, *C. d.* var. *ulcerans* produces diphtherial toxin (*see* Fig. 16). Lysogenic, toxinogenic strains occur naturally. They synthesize a phage-restricting enzyme different from that occurring in $C7_s(-)^{tox^-}$. Their walls are essentially like those of the type species (Arden, *unpublished data*). They are neuraminidase producers.

C. pseudotuberculosis (*ovis*), like *C. d.* var. *ulcerans*, appears to be closely related to *C. diphtheriae*. It shares susceptibility to a number of the same phages, and when lysogenized with a tox^+ phage it synthesizes diphtherial toxin (*see* Fig. 16). It sometimes does infect human beings (28) about as frequently as *C. diphtheriae* (93) and *C. d.* var. *ulcerans* (117) infect animals. Although an *ovis* exotoxin has long been known and methods for its production have several times been described, except for its effects on mice and guinea pigs and its neutralization by an antiserum, nothing is known of its actual nature (140, 141, 186). Soucek described a sphingomyelinase associated with *ovis* extracts. About this, one would like to see more data (283). The walls of *ovis* strains are similar to those of *C. diphtheriae*. *C. ovis* produces a cord factor (45), the structure of which has not yet been determined. *C. pseudotuberculosis* (*ovis*) is reported to ferment lactose (36); however, none of the strains encountered by us has been lactose positive. To resolve this contradiction is important because no other true corynebacteria are lactose-positive. Strains of this bacterium are good neuraminidase producers.

From a limited examination in this laboratory, it appears that *C. bovis*, *C. equi*, *C. kutscheri*, "*C. belfanti*," *C. pseudodiphthericum* (*hofmanni*), *C. renale*, and *C. xerosis* are insensitive to the corynebacteriophages at our disposal.

Possible Candidates for the Genus *Corynebacterium*

Whether *Microbacterium flavum* and *M. thermosphactum* will turn out to be close relatives of *C. diphtheriae* remains to be seen. They have a murein pattern similar to that of *C. diphtheriae* (273), but the nature of their wall polysaccharides remains to be elucidated. At this time nothing is known of their antigenic structure or of their bacteriophages (*see* also 62*a*). *Brevibacterium* (36) may, in part, also be a candidate (318).

Species to be Dropped from the Genus *Corynebacterium*

The following strains of so-called corynebacteria should be removed from the genus *Corynebacterium* on the grounds that they have too little in common with the type species and on the basis of properties here listed. A new genus for each should be found.

C. pyogenes. Walls contain no DAP; arabinogalactan is not present but in its place is a rhamnosyl polymer which cross-reacts with antisera prepared versus group G streptococci. Metabolism is that of lactic acid bacteria (26).

Propionibacterium: C. acnes, C. parvum. Walls contain LL DAP, no arabinogalactan, no mycolic acids (209, 218, 309). Their specific bacteriophages are without effect on true corynebacteria. They produce a phenol oxidase not found in *Mycobacterium, Corynebacterium,* or *Nocardia* (Barksdale, B. Beaman, and L-V. Beaman, *unpublished data*).

The plant pathogenic "corynebacteria," *C. tritici, C. betae, C. flaccumfaciens,* and *C. poinsettiae,* have no place among the true corynebacteria because in their properties they are too far from the description of the type species; e.g., the major diamino acid of their walls is either diaminobutyric (in the case of *C. tritici*) or ornithine (in the case of the others). They all lack arabinogalactan, and no mycolic acids have been described for them (234, 235).

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